University of Alberta

Nitric Oxide: A Novel Mediator of Agonist-Induced Growth Hormone Secretion

By

Aubrey David Uretsky ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

In

Physiology and Cell Biology

Department of Biological Sciences

Edmonton, Alberta, Canada

Fall 2001

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-69817-3

Canada

University of Alberta

Library Release Form

Name of Author: Aubrey David Uretsky

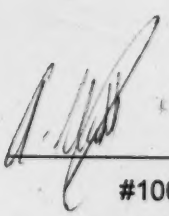
Title of Thesis: Nitric Oxide: A Novel Mediator of Agonist-Induced Growth Hormone Secretion

Degree: Master of Science

Year this Degree Granted: 2001

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly, or scientific research purposes.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.



#1004, 10045-118 street
Edmonton, Alberta, Canada
T5K 2K2

July 24, 2001

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Nitric Oxide: A Novel Mediator of Agonist-Induced Growth Hormone Secretion submitted by Aubrey David Uretsky in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Cell Biology.

J.P. Chang, Supervisor

J.I. Goldberg, Committee Member

July 23, 2001

T.L. Krukoff, Committee Member/External Examiner

Breathe, breathe in the air.

Don't be afraid to care...

...Long you live and high you fly

And smiles you'll give and tears you'll cry

And all you touch and all you see

Is all your life will ever be.

From "Breathe" by Pink Floyd

Roger Waters, David Gilmour, and Rick Wright.

Abstract

Nitric oxide (NO) is a gaseous signaling molecule that has been shown to regulate the secretion of many hormones. If and how NO plays a role in regulating growth hormone (GH) secretion from goldfish pituitary cells was investigated. Immunocytochemical studies with antibodies against mammalian isoforms of nitric oxide synthase (NOS) revealed the presence of a NOS-like protein in dispersed pituitary cells. Treatment of cells with NO donors increased GH secretion, and this increase was blocked by NO scavengers, somatostatin, and soluble guanylate cyclase inhibitors. Treatment with inhibitors of NOS and soluble guanylate cyclase, and NO scavengers inhibited the GH release response to two endogenous gonadotropin-releasing hormones known to increase GH secretion. Other results suggest that dopamine-, but not pituitary adenylate-cyclase activating polypeptide-stimulated GH release involves NO. In summary, NO appears to be involved in mediating the GH responses to select neuroendocrine regulators through a cGMP signaling mechanism.

Acknowledgements

I would like to begin by thanking my supervisor John Chang, who's willingness to take me on as a graduate student despite my aspirations to study medicine in conjunction with science, is something I am eternally grateful for. John, your support, teaching, and friendship (oh, and cooking!) are things that will stay with me for life. I would also like to thank my committee members, to Jeff Goldberg, the man who gave me my first taste of research and sparked an interest in me that will never go away; thank you for your support and insight on matters of science, sports, and life in general. To Dr. Tereasa Krukoff, thank you for making the time to serve on my committee. As well, to the defense chair, Dr. Bruce Hemming, thank you for ensuring the defense went smoothly and quickly.

Over the past few years, I have been fortunate enough to work with some of the brightest, and most disturbed minds in the department of biological sciences at the University of Alberta. Whether engrossed in scientific conversation, playing a little hacky sac, going for coffee, or attempting to figure out the best way to cram as much vodka as possible into an intact watermelon, we've had a lot of fun, you guys are the best. To Brian Weiss, Shandra Doran, Warren Yunker, Calvin Wong, Kevin Friesen, Jim Johnson, Geoff Boddy, and the rest of the 5th floor crew, you guys have made my time here as much fun and intellectually challenging as I could have ever hoped for. Thank you all for the good times. A special thanks also goes out to Phil Davis for topping up a few n's for me in my thesis writing time of need.

I would also like to thank my family for all of their support during my graduate student days, and for continuing that support while I chose to finish my MSc. while attending medical school. I couldn't have done it without you guys. Mom, Dad, Jason, and Sari, thanks guys, you are the greatest and most loving family I could ever be so lucky to be a part of.

Last, and most importantly, I consider myself the luckiest man alive for one reason. My soon to be bride, Shira, is with me after many years apart. Shira, your love and support are a source of strength for me everyday and none of this would have been possible without you.

Thank you to everyone, you have all made this thesis possible and more importantly, you have all, in your own ways, made me who I am today.

Table of Contents

1. Introduction 1

1.1 Structure and biosynthesis of nitric oxide.....1

1.2 NOS isoforms.....3

1.3 NO's signaling cascade.....6

1.4 NO as a neuroendocrine regulator.....11

1.5 The control of growth hormone secretion in mammals.....12

1.6 Growth hormone regulation by NO in mammals.....13

1.7 NO and pituitary hormone release in lower vertebrates.....17

1.8 Growth hormone secretion in the goldfish (*Carassius auratus*).....18

1.9 Goals and objectives.....21

2. Materials and methods 23

2.1 Animals.....23

2.2 Drugs.....23

2.3 Pituitary cell dispersion.....24

2.4 Immunocytochemistry.....25

2.5 Static incubation hormone release studies.....28

2.6 Perifusion studies on hormone release.....28

2.7 Cyclic GMP assay.....30

3. Results 31

3.1 NO effects on basal growth hormone secretion.....31

3.2 NO mediation of GnRH-stimulated growth hormone release.....44

3.2.1 Effects of NOS inhibitors on GnRH-stimulated growth.....44
hormone release
3.2.2 Effects of NO scavengers on GnRH-induced growth.....55
hormone release
3.2.3 Possible involvement of sGC activity.....60
3.3 NOS localization in pituitary cells.....65
3.4 NO involvement in the growth horomne-release stimulating activity of other known growth hormone secretagogues.....68
4. Discussion 83
4.1 NO stimulates growth hormone secretion.....83
4.2 NO is required for GnRH stimulation of growth hormone secretion...85
4.3 NO actions on growth hormone secretion are mediated by cGMP...88
4.4 NO is not involved in unstimualted growth hormone secretion.....90
4.5 NO can be produced endogenously in goldfish pituitary cells.....91
4.6 Is iNOS the enzyme present in goldfish pituitary cells?.....95
4.7 NO is also involved in the regulation of dopamine-stimulated growth hormone secretion via a cGMP-mediated mechanism.....97
4.8 NO is not likely involved, but cGMP may be involved in PACAP stimulation of growth hormone release.....100
4.9 So what role does NO play in the control of growth hormone secretion?.....101
4.9.1 NO in GnRH signal transduction.....101
4.9.2 NO in dopamine and PACAP signal transduction.....106

4.10 Summary.....109

Endnotes 111

Literature cited 112

Appendix A- Alternate methods 124

A-1 Nitrite assay.....124

A-2 Western blot analysis 124

A-3 DAF-2 imaging 125

List of Figures

1. Schematic representation of the three known mammalian isoforms of NOS.
2. Different oxidative states of NO and their intracellular actions.
3. Representation of the known GH secretion pathways in goldfish somatotropes.
4. Somatotrope cell identification features.
5. NO can stimulate GH secretion in 2-hour static culture.
6. NO can stimulate GH secretion in cell column perifusion.
7. NO scavenger abolishes GH secretion in response to NO.
8. SRIH abolishes GH secretion in response to NO.
9. cGMP can stimulate GH secretion.
10. sGC activity is required for NO stimulation of GH secretion.
11. GH response to GnRHs is unaffected by a mammalian bNOS inhibitor.
12. GnRH stimulation of GH release is abolished by broad spectrum NOS inhibition.
13. GnRH stimulation of GH release is abolished by iNOS inhibition.
14. NOS activity is required for cGnRH-II stimulation of GH.
15. NOS activity is necessary for sGnRH stimulation of GH.
16. A NO scavenger inhibits cGnRH-II stimulation of GH.
17. A NO scavenger inhibits sGnRH stimulation of GH.
18. sGC activity is required for cGnRH-II stimulation of GH.
19. sGC activity is required for the GH release response to sGnRH.
20. bNOS-like immunoreactivity in somatotropes and gonadotropes.
21. iNOS-like immunoreactivity in somatotropes and gonadotropes.

22. bNOS inhibition has no effect on DA D1- and PACAP-stimulated GH release in static culture.

23. bNOS inhibition has no effect on DA D1-stimulated GH secretion in perifusion.

24. Broad spectrum NOS inhibition significantly reduces the GH release response to DA D1 stimulation in static culture.

25. Broad spectrum NOS inhibition significantly reduces the GH release response to DA D1 stimulation in perifusion.

26. iNOS inhibition reduces the GH-releasing effect of DA D1-stimulation, but does not affect PACAP-stimulated GH release in static culture.

27. GC inhibition abolishes the GH release response to both PACAP and a DA D1 agonist.

28. Guanylate cyclase inhibition has no effect on DA D1-stimulated GH secretion in perifusion.

29. Schematic representation of the possible mechanisms by which NOS activity, NO generation, and other signaling components regulate the GH secretion induced by sGnRH and cGnRH-II.

30. Schematic representation of possible mechanisms by which NOS activity, NO generation, and other signaling components selectively regulate the GH secretion induced by DA and PACAP.

List of Abbreviations

1400W	N-(3-Aminomethyl) benzylacetamidine dihydrochloride
7-NI	7-nitroindazole
AA	Arachidonic acid
AGH	Aminoguanidine hemisulfate
bNOS	Brain nitric oxide synthase
$[Ca^{2+}]_c$	Cytosolic calcium concentration
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
cGnRH-II	Chicken gonadotropin-releasing hormone-II
DA	Dopamine
dbcGMP	Dibutyryl guanosine 3':5'-cyclic monophosphate
eNOS	Endothelial nitric oxide synthase
GH	Growth hormone
GHRH	Growth hormone-releasing hormone
iNOS	Inducible nitric oxide synthase
IP_3	Inositol trisphosphate
LY 83583	6-Anilino-5, 8-quinolinequinone
NO	Nitric oxide
ODQ	1-H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one
PACAP	Pituitary adenylate cyclase-activating polypeptide
PKA	Protein kinase A
PKC	Protein kinase C
PTIO	2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
sGC	Soluble guanylate cyclase
sGnRH	Salmon gonadotropin-releasing hormone
SKF 38393	(±)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride
SNAP	S-nitroso-N-acetylpenicillamine
SNO	S-nitrosothiol
SNP	Sodium nitroprusside
SRIH	Somatostatin

1. Introduction

Nitric Oxide (NO) is involved in a wide range of physiological actions. Although its biosynthetic precursor, L-arginine, had been shown to stimulate the secretion of some hormones (Alba-Rothet al., 1988; Ghigo et al., 1996), it was not until 1987 that the possibility of NO as the affector molecule was carefully considered. The first identified role of NO as a signaling molecule was as the elusive endothelial-derived relaxation factor produced from vascular endothelial cells (Moncada et al., 1991). Since this first discovery of endogenous action, NO has been implicated in many different physiological functions. These actions include blood pressure regulation (Ledingham and Laverty, 1997), reproduction (Bonavera et al., 1993; Moretto et al., 1993; McCann et al., 1998), immune response (Mayer and Hemmens, 1997), neurotransmission (Beck et al., 1999), protein synthesis (Curran et al., 1991), cell signaling (Beck et al., 1999), intracellular Ca^{2+} regulation (Willmott et al., 1996; 2000) and more. Over the last 10 years, NO's involvement in neuroendocrine regulation has been investigated. NO has been implicated in the regulation of hormone secretion from the hypothalamic-pituitary axis and from many other endocrine tissues (Brunetti, 1996).

1.1 Structure and biosynthesis of nitric oxide

NO is formed by a reaction that converts L-arginine into L-citrulline and NO (Brunetti, 1996). This reaction is catalyzed by a family of enzymes known as nitric oxide synthases (NOSs). Furthermore, this reaction requires the presence

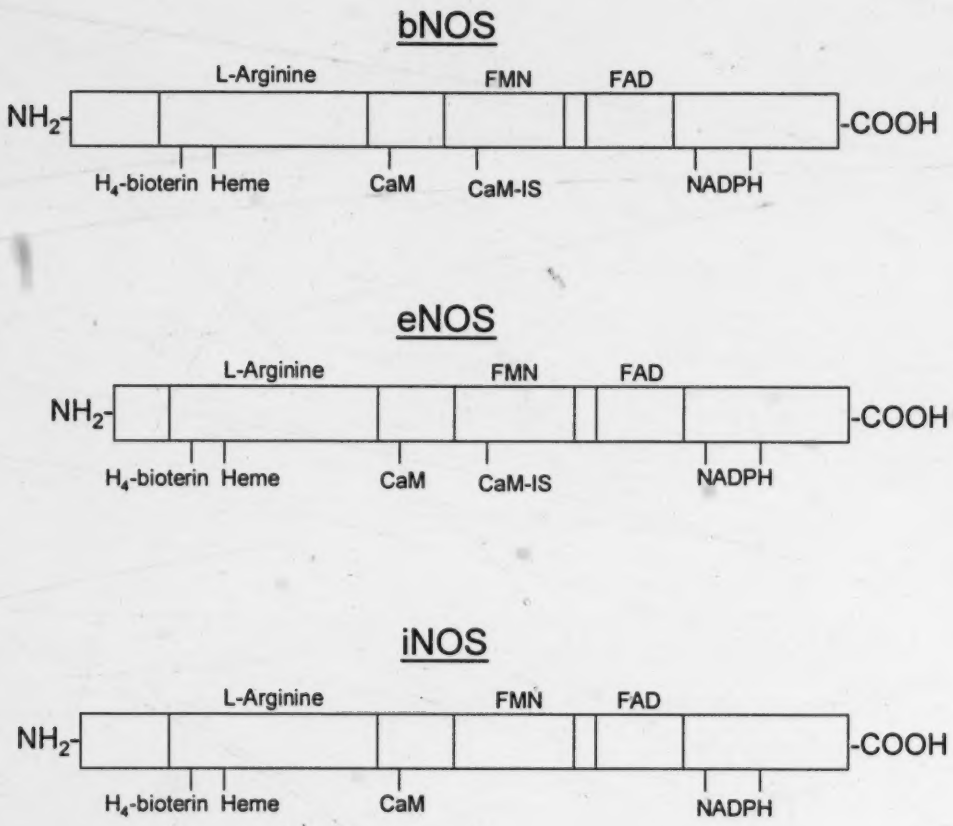
of many cofactors including NADPH, oxygen, heme, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), calmodulin (CaM) and tetrahydrobiopterin (H_4biopterin) (McDonald and Murad, 1996).

Once formed, NO is a gas that is rapidly degraded to nitrate and nitrite, with a half-life of about 4-10 s in aqueous solution (McCann, 1996). NO does not act as a classical intercellular messenger that binds a specific membrane bound receptor to exert its effects. Instead it freely diffuses across membranes due to its low molecular weight and relatively high solubility and hydrophobicity (McDonald and Murad, 1996). As a free radical, its biological effects are determined by its chemical reactivity (Mayer and Hemmens, 1997). NO's highly reactive nature can thus determine its availability for both intra- and inter-cellular action. For example, if NO is diffusing into or out of a cell, this high reactivity increases the chance of a reaction with a molecule near the cell membrane as opposed to a target situated closer to the middle of a cell. Thus, NO diffusing across a cell membrane would likely act on plasma membrane bound or soluble target sites rather than a nuclear site. Furthermore, NO binds with high affinity to the iron centre of hemoglobin and other heme proteins, and to thiol groups in proteins, such as albumin in blood. Such binding to proteins found in blood and other extracellular fluids can limit its practical diffusion potential, as well as its ability to travel to distant target sites (McDonald and Murad, 1996). In addition, these factors make the location of NOS within cells an important factor in determining what NO will react with. Taken together, these properties almost completely exclude NO from functioning as a classical hormone that is carried in

the blood to its distal target tissues. This leaves intracrine, autocrine, and paracrine roles as the most likely modes of action for this unusual signaling molecule.

1.2 NOS isoforms

There are three well-characterized isoforms of NOS in mammals. All of these isoforms are strictly dependent on CaM, which activates the enzyme by facilitating electron transfer within the reductase region (Sessa, 1994). Two of these isoforms, collectively known as cNOSs, are constitutively present in cells. The third isoform, iNOS, is only present when its synthesis is induced by appropriate stimuli. The homology of the three isoforms is only about 50-60%, while the homology of a given isoform between species can be as much as 85-92% (Murad, 1999). Despite only a 50-60% homology, all three isoforms of NOS are similar in respect to the layout of their activation and catalytic domains (Fig. 1; Hattori et al., 1994). They all have a C-terminal reductase domain containing the binding sites for FAD, FMN, and NADPH. They also all have a N-terminal oxygenase domain containing bound heme and the H_4biopterin binding site, which is essential for NADPH-dependent O_2 activation (Mayer and Hemmens, 1997). The CaM binding site of these enzymes is at the N-terminal edge of the reductase domain. One major difference between the iNOS and the cNOS isoforms is that the cNOS isoforms contain a CaM inhibitor sequence in the FMN domain which renders iNOS activity insensitive to increases in cytosolic free



Adapted from Mayer and Hemmens, 1997; Knowles and Moncada, 1994; Sessa, 1994.

Figure 1. Schematic representation of the three known mammalian isoforms of NOS. Diagram illustrates the important binding sites for regulatory agents and the CaM inhibitor binding sequence (CaM-IS), which is present in only the bNOS and eNOS isoforms.

calcium concentration ($[Ca^{2+}]_c$; Michel and Feron, 1997; also see discussion on activation of cNOS and iNOS below).

The cNOSs, brain (b) or neuronal (n) NOS (NOS 1; type I NOS) and endothelial (e) NOS (NOS III; type 3 NOS), were first discovered in brain/neuronal and endothelial tissue, respectively. The brain isoform is a 160 kDa protein normally found in the soluble fraction of cells (Hattori et al., 1994). eNOS is a 135 kDa protein usually found in the particulate fraction of cells and has been shown to be associated with caveolae in the plasma membrane (Murad, 1999). These two enzymes, like iNOS, require CaM for activation, but unlike iNOS, require increases in $[Ca^{2+}]_c$ to bind CaM and become active. It was further determined, through studies exchanging the CaM binding sequences between the isoforms, that the CaM inhibitory sequence of the cNOSs is displaced only by Ca^{2+}-bound CaM, thus activating the enzyme (Venema et al., 1996). The major increase in activity of these enzymes is typically seen between 100-500 nM $[Ca^{2+}]_c$ (Schmidt et al., 1992). Interestingly, these levels of $[Ca^{2+}]_c$ are also able to activate Ca^{2+}/CaM Kinase-II, which can in turn, phosphorylate cNOSs leading to their inactivation, thus representing a possible negative regulatory mechanism on cNOS activity. cNOSs can also be phosphorylated by other Ca^{2+}-activated kinases such as protein kinase A and C (PKA, PKC) leading to enzyme inactivation (Sessa, 1994).

The third isoform, inducible (i) NOS (NOS II; type 2 NOS), was first discovered in macrophages. It is not constitutively present in cells, but its synthesis is induced by cytokines and other immune response elements. The

CaM inhibitor sequence in the FMN binding domain of the constitutive isoforms is absent in the iNOS sequence (Mayer and Hemmens, 1994), as a result iNOS binds CaM (not bound to Ca^{2+}) at even the lowest physiological levels of $[Ca^{2+}]_c$ encountered in vivo. Thus, iNOS, when formed, is always active. Due to the fact that iNOS must be synthesized in cells in response to immune response elements, the production of NO after the induction of iNOS synthesis is delayed by anywhere from a few hours to eighteen hours to allow for enzyme production (Sparrow, 1995). However, once translated, iNOS produces about ten-fold more NO than the cNOS isoforms.

While mammalian forms of the three NOSs are well characterized (Knowles and Moncada, 1994), the only teleost NOS enzyme to be completely purified and cloned is a carp iNOS (Saeij et al., 2000), while a goldfish iNOS has been partially sequenced (Laing et al., 1996). The carp iNOS contains binding sites for all the same cofactors as the mammalian iNOS and has 57% sequence homology to the mammalian isoform.

1.3 NO's signaling cascade

Almost all intracellular components are potential targets for NO (McDonald and Murad, 1996). Most of NO's interactions with enzymes result in inactivation. One major exception is soluble guanylate cyclase (sGC), which is activated by NO. sGC is the major target for physiological effects of NO. At nanomolar concentrations, NO activates sGC by binding to its heme group, thereby activating the enzyme and stimulating the formation of cGMP by several hundred

fold (Mayer and Hemmens, 1997). cGMP then acts on many targets, including cGMP-dependent protein kinases (McDonald and Murad, 1996). These kinases are selectively, but not solely activated by cGMP, as cAMP can also bind and activate these enzymes. There are also a number of ion channels, including Ca^{2+} channels, which are potential downstream targets gated by cGMP. These channels often have a single cGMP regulatory site that controls Ca^{2+} flux into the cell, but it is not always clear if the channel is directly regulated by cGMP, or indirectly regulated through cGMP activation of the cGMP-dependent protein kinases mentioned above (McDonald and Murad, 1996).

In addition to these effects on sGC, NO itself has been implicated in causing changes in $[Ca^{2+}]_c$ (Willmott et al., 2000). Studies have shown that NO increases $[Ca^{2+}]_c$ either directly by stimulating the ryanodine receptor on endoplasmic reticulum, or indirectly by promoting the formation of the Ca^{2+}-mobilizing second messenger, cyclic ADP-ribose (cADPR) (Willmott et al., 1996). Whether or not NO stimulation of cADPR formation is exclusively carried out through cGMP is currently under debate, but it is clear that increases in cADPR have been measured in PC12 cells after treatment with NO donors and this increase is blocked by a specific cGMP-dependent protein kinase I blocker (Clementi et al., 1996). cADPR is formed by the enzyme ADP ribosyl-cyclase which can be found in both membrane-bound and soluble forms (Guse, 1999). The soluble form is sensitive to cGMP (Graeff et al., 1998). cADPR appears to act on type 2-ryanodine receptors to cause Ca^{2+} release from ryanodine-sensitive intracellular Ca^{2+} stores. The proposed mechanism is thus as follows.

NO-activated sGC produces cGMP, which then leads to an increase in the activity of a cGMP-dependent protein kinase. Activated cGMP-dependent protein kinase phosphorylates ADP-ribosyl cyclase, leading to the production of cADPR, which acts on type 2-ryanodine receptors on the endoplasmic reticulum culminating in the release of Ca^{2+}.

While the activation of sGC is the most commonly referred to signal transduction pathway for NO, many more possibilities exist. To understand why these other possibilities exist, it is important to first understand that NO does not refer only to a single species, but to a number of different redox states of nitrogen. These different states are the free radical NO•, nitrosonium ion (NO^+), and nitroxyl anion (NO^-; Gow and Ischiropoulos, 2001; Fig. 2). The fact that NO can exist as NO^+ allows it to be a potent nitrosating agent. While it is true that NO^+ will nitrosate water to produce nitrite, it is also true that in the presence of other nucleophiles, NO^+ will react with them. One of the most common intracellular nucleophiles is reduced thiols with which NO^+ can produce S-nitrosothiols (SNO). Reduced thiol groups also can react with the free radical NO• to produce SNOs, thus making SNOs a very likely end product of NO generation within cells. In addition to thiols, many other proteins have been shown to be S-nitrosylated in vivo. Such proteins include hemoglobin (Jia et al., 1996), the ryanodine receptor (Xu et al., 1998) and other components of the classical intracellular signal transduction mechanisms, such as G-proteins and kinases (Gow and Ischiropoulos, 2001). The ryanodine receptor (a calcium channel protein) contains 84 thiol groups and S-nitrosylation of merely one

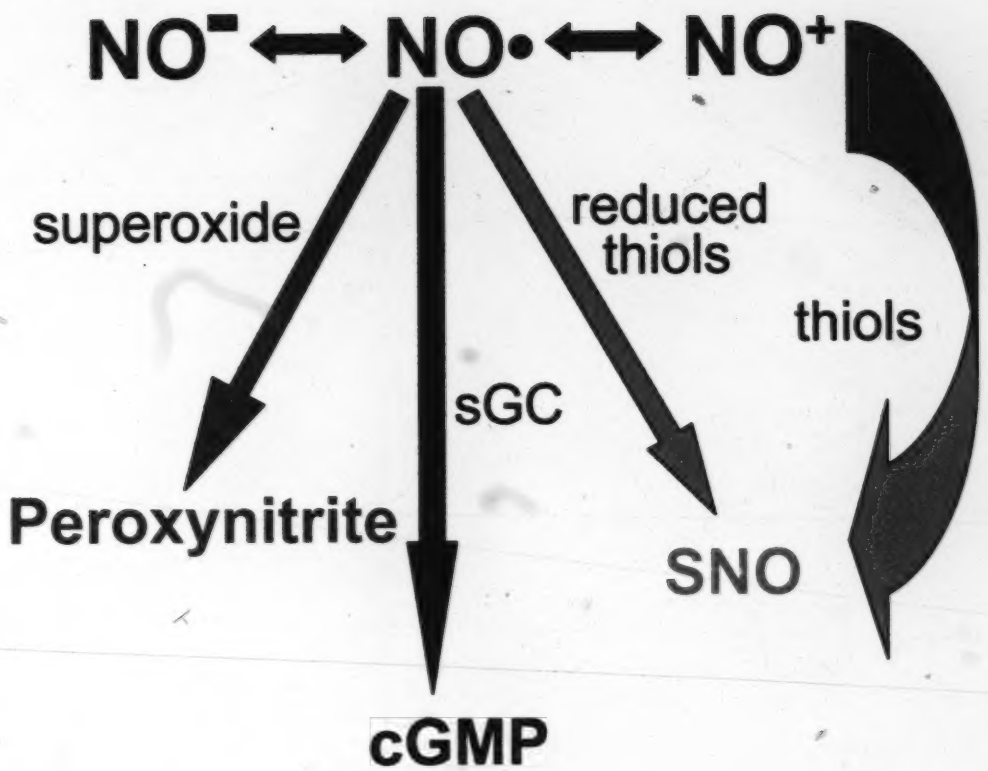


Fig. 2 Schematic representation of the different oxidative states of nitric oxide, their most likely intracellular reactions, and subsequent formation of products that modify cellular activity.

cysteine residue within this receptor can lead to Ca^{2+} channel opening (Gow and Ischiropoulos, 2001).

Consequent to its free radical form, NO can also react with superoxides to produce peroxynitrite. This occurs via reactions with cysteine residues as well. In vivo, peroxynitrite usually quickly decomposes to the final end product nitrite (Gow and Ischiropoulos, 2001).

Due to the fact that there are so many possible reactions for NO within and around cells, the important question becomes which one of these many reactions will be the fate of NO in any one cell at any one time. The first element that will determine what NO will react with is where the NO is produced and what is in its immediate vicinity. The rate of reaction with the local molecules is another key element in determining what NO will react with. The fastest known biological reaction is the combination of NO with superoxide to form peroxynitrite (Gow and Ischiropoulos, 2001). However, under non-pathological conditions this is likely a negligible reaction because the concentration of superoxide is kept very low and is tightly regulated by cells. The rate of reaction with metals is also a very fast reaction, but again, the levels of metals are generally low in cells, except for the heme-iron proteins like sGC. Thus, although reaction with reduced thiols may be one of slowest reactions which NO undergoes, it may be the most prevalent one due to the fact that reduced thiols are generally very abundant in cells, often in concentrations of 5-10 mM (Gow and Ischiropoulos, 2001). Furthermore, if one considers sGC more closely it can be noted that sGC can be activated by NO via binding to the heme-iron moiety, but sGC can also be acted

on by SNOs through interactions with the two conserved cysteine residues close to the heme.

Overall, NO seems most likely to act through the activation of sGC and subsequently through cGMP, but this is only one of many possible sites of action of NO. NO has also been shown to reversibly bind and subsequently inhibit cytochrome oxidase in mitochondria which leads to an inhibition of mitochondrial respiration (Richter et al., 1997). NO has also been demonstrated to be lethal to cells at high concentrations. This cytotoxicity results from NO_3^--induced increases in $[Ca^{2+}]_c$. NO_3^- can possibly mobilize cytosolic calcium to lethal levels by stimulating the specific ADP-ribose-dependent release pathway in mitochondria leading to toxic levels of Ca^{2+} release (Richter et al., 1997). Furthermore, NO has been implicated as an integral part of the apoptotic pathway in many cell types (Szabolcs et al., 2001). These complexities of multiple actions make the study of NO both challenging and extremely exciting.

1.4 NO as a neuroendocrine regulator

Among NO's many functions, its role as a neuroendocrine regulator is one of the most exciting fields of study. NO has been demonstrated to participate in the secretory control of multiple hormones in mammals. Many of these hormones are associated with the hypothalamic-pituitary axis (Brunetti, 1994). NO and its precursor, L-arginine, have been shown to participate in the regulation of secretion of hormones such as prolactin (Vankelecom et al., 1997;

Duvilanski et al., 1996), luteinizing hormone (Bonavera et al., 1993; McCann et al., 1998), adrenocorticotropic hormone (Volpi et al., 1996; Turnbull and Rivier, 1996), somatostatin (SRIH; somatotropin-release inhibitory hormone; Alba-Roth et al., 1988; Ghigo et al., 1996), gonadotropin-releasing hormone (GnRH; Moretto et al., 1993), vasopressin, corticotropin (Brunetti, 1994), growth hormone-releasing hormone (GHRH; Kato, 1992) and growth hormone (GH; Rettori et al., 1994; Tena-Sempere et al., 1996).

1.5 The control of growth hormone secretion in mammals

The neuroendocrine control of GH secretion from the mammalian anterior pituitary somatotropes has been extensively investigated in the rat. GH secretion is controlled by a complex system of neuroendocrine factors that mostly lead to regulation of the two major, direct, GH-controlling factors in the mammal, GHRH and SRIH (Müller et al., 1999; Frohman et al., 1992). GHRH and SRIH exert stimulatory and inhibitory control, respectively, at the level of the somatotrope. Although somatotropes are also acted on by other circulating and local hormones such as pituitary adenylate cyclase-activating poly-peptide (PACAP), thyrotropin-releasing hormone (TRH), dopamine (DA), corticotropin-releasing hormone, neurotensin, vasointestinal peptide, neuropeptide Y, and galanin, GHRH and SRIH are believed to be the two major factors regulating GH secretion (Müller et al., 1999).

GHRH receptors have been located on somatotropes in rats. GHRH binding to these G-protein coupled receptors has functionally been linked to

increases in GH secretion, GH gene transcription and biosynthesis, and somatotrope proliferation (Frohman et al., 1992). Evidence implicating cAMP as the second messenger system of GHRH has been provided by studies showing a dose-dependent stimulation of adenylate cyclase activity and cAMP production in somatotropes. GHRH also elevates $[Ca^{2+}]_c$ in somatotropes. Although it has been proposed that cAMP actions on plasma membrane Ca^{2+} channels mediate this effect of GHRH, whether extracellular Ca^{2+} entry and/or efflux from intracellular stores are the source of this rise remains controversial. SRIH effects at the level of the pituitary somatotrope have been studied and results indicate that SRIH causes a dose-dependent decrease in $[Ca^{2+}]_c$ (for a review, see Müller et al., 1999). In addition, SRIH can regulate GHRH action by binding to a SRIH receptor that is negatively coupled to adenylate cyclase through a G_i protein.

1.6 Growth hormone regulation by NO in mammals

With a growing number of studies implicating NO in the control of many neurohormones, a role for NO in the regulation of GH secretion from somatotropes has also been proposed. However, the exact role that NO plays in the regulation of GH release is controversial.

A study in rats has localized bNOS immunoreactivity to many hypothalamic nuclei important for neuroendocrine regulation of GH, such as the arcuate nucleus, the preoptic area, and the median eminence. Furthermore, this study demonstrated that increases in cGMP levels coincide with increases in NO concentrations in these hypothalamic nuclei (Bhat et al., 1996). In rats, the

median eminence is the secretion site for both SRIH and GHRH, while the arcuate contains GHRH neuronal cell bodies and SRIH input (Müller et al., 1999). Taken together with these anatomical characteristics of the SRIH and GHRH neuronal system, the findings on hypothalamic NO activities imply that NO can control the secretions of SRIH and GHRH. Therefore, NO can indirectly alter GH release from the pituitary through the control of hypothalamic factors. Further evidence to support a possible role for NO in the control of GH secretion is evident in studies implicating a role for L-arginine in the stimulation of GH release (Müller et al., 1999). Since L-arginine is the precursor for NO, these authors postulate that L-arginine stimulation of GH secretion is through the production of NO. While this theory is logical, it has been observed that the GH-releasing ability of L-arginine is likely due to a non-NO mediated suppression of SRIH (Alba-Roth et al., 1988). Other studies that demonstrate L-arginine stimulation of GH release, also demonstrate that treatment with NOS inhibitors did not reduce GH secretion in response to L-arginine and thus conclude that NO is not the effector molecule (Fisker et al., 1999).

The role NO plays at the level of the pituitary is also controversial. Studies have localized NOS immunoreactivity to mainly two cell types in the anterior pituitary of the rat, the follicle-stellate cells and gonadotropes (Yamada et al., 1997). No NOS-immunoreactivity has been observed in somatotropes of the rat, suggesting that rat somatotropes cannot produce NO. However, the idea that NO produced in follicle-stellate cells and/or gonadotropes could diffuse into somatotropes to exert effects on GH release via a paracrine manner remains a

distinct possibility. Support for a role for NO in GH release is evident in that cGMP has been shown to stimulate GH release from rat anterior pituitary cells (Hartt et al., 1995), and cGMP staining has been both co-localized with NOS staining in gonadotropes, as well as in other unidentified cells in the rat anterior pituitary (Yamada et al., 1997). Furthermore, increases in cGMP staining were seen following treatment of anterior pituitary cells with NO donors. Unfortunately in this study, no effort was made to determine if the identity of the non-gonadotropes that were cGMP-immunoreactive after addition of a NO donor were indeed GH cells.

More direct in vitro and in vivo studies investigating the role of NO on GH release have yielded puzzling results. An early study by Kato and colleagues (1992) reports an inhibitory role for NO. In this study, NO scavengers were able to potentiate the GHRH-induced GH secretion, while NO donors suppressed the GHRH-induced GH secretion in vitro. In contrast to these results, later studies have shown an opposite effect (Rettori et al., 1994; Tena-Sempere et al., 1996). In one such study, GHRH was unable to induce GH secretion in vivo or in vitro after treatment with NOS inhibitors (Tena-Sempere et al., 1996). In agreement with this data, another study has shown that GHRH-stimulated GH secretion can be suppressed in vivo by pre-treatment with a NOS inhibitor (Rettori et al., 1994). These latter studies thus implicate NO as a stimulatory factor on GH release. Further evidence to support this stimulatory role for NO at the level of the pituitary somatotrope is seen in a study implicating cGMP as a stimulatory regulator of GH secretion in vitro from rat anterior pituitary cells (Hartt et al.,

1995). Regardless of whether NO is stimulatory or inhibitory to GH release, none of the above mentioned studies can satisfactorily account for how the production of NO can be achieved. While available evidence suggests that NOS protein is not in GH cells and is only present in follicle-stellate and gonadotropin cells, a paracrine mode of action is the only possibility. However, none of these studies address how the production of NO in these two cell types can be brought about by GHRH or other regulatory factors of GH secretion.

Whether NO inhibits or stimulates GH secretion has remained in the forefront as more evidence in support of both sides of this debate has recently come to light. In addition, a new controversy over the mechanism of these NO actions has also arisen. Tsumori and colleagues (1999) propose that NO inhibits TRH-induced GH secretion from GH_3 cells in a cGMP-dependant manner. Unfortunately the data used to support this argument is somewhat weak. They showed that a known inhibitor of NOS enhanced basal and TRH-induced release of GH in vitro, and that a cGMP antagonist also increased GH release. However, they failed to explain why a NO donor molecule had no effects on either basal or TRH-induced GH secretion. It appears counterintuitive that the inhibition of NO production had such a profound effect while addition of NO had no effect. In addition, application of a NO scavenging molecule was only able to moderately decrease the GH-release response to the first of two consecutive pulses of TRH in a temporal study. Furthermore, the only evidence presented to support the conclusion that cGMP is involved in this inhibition, is that a cGMP antagonist mildly increased basal GH secretion. The other side of this argument, implicating

NO as a stimulatory modulator of GH secretion, is evident in a paper by Pinella and colleagues (1999). In this paper the authors claim that NO stimulates GH secretion independent of both cGMP and Ca^{2+}. While results presented are mostly convincing, this study does not adequately investigate the role of intracellular Ca^{2+} stores as a possible source of Ca^{2+} and never actually measures $[Ca^{2+}]_c$. Furthermore, the magnitude of the GH secretory response to NO donors were extremely variable and this variability was never addressed. Regardless of their shortcomings, these two studies add more fuel to the ongoing debate as to what role NO plays in the control of GH release in the rat.

1.7 NO and pituitary hormone release in lower vertebrates

Very few studies have been performed on lower vertebrates to determine the involvement of NO in the control of pituitary hormone secretion. A study on frog pituitaries shows a probable role for NO in the regulation of GnRH effects on the pituitary (Gobbetti and Zerani, 1998). Studies performed on the male newt indicate that NO and cGMP up-regulate both basal and GnRH-induced pituitary gonadotropin secretion in vitro (Gobbetti and Zerani, 1999). In addition, a more recent study has implicated endogenous NO production in the stimulation of melanotropin secretion from the pars intermedia of *Xenopus laevis* (Allaerts et al., 2000). This study also localized both bNOS and iNOS immunostaining in cultured pars intermedia cells. These data suggest that NO may also play a neuroendocrine regulatory role in lower vertebrates.

1.8 Growth hormone secretion in the goldfish *(Carassius auratus)*

Among neuroendocrine regulatory systems in lower vertebrates, the control of GH secretion in the goldfish is among the best characterized, both in terms of the involvement of multiple regulatory factors and the interactions of their respective intracellular signal transduction pathways (Peter and Chang, 1999). Of the many known factors that directly stimulate GH secretion in goldfish, two endogenous GnRHs (salmon (s)GnRH and chicken (c)GnRH-II) and DA appear to be the major regulators in terms of their consistency in being able to stimulate GH secretion at most times of the seasonal reproductive cycle. Activation of GnRH and DA D1 receptors on goldfish somatotropes leads to GH secretion via distinct signal transduction pathways (Fig. 3; Peter and Chang, 1999). GnRH-induced responses are mediated by protein kinase C (PKC) and have been shown to involve both entry of extracellular Ca^{2+} and mobilization of Ca^{2+} from intracellular Ca^{2+} pools (Johnson and Chang, 2000). In addition, the two endogenous GnRHs have been demonstrated to utilize pharmacologically distinct Ca^{2+} stores in these cells (Johnson, 2000). Whether the Ca^{2+} signals generated by the two GnRHs in somatotropes are distinct, as in the situation in gonadotropin cells (Johnson et al., 1999), remains to be determined. DA D1 actions are mediated via cAMP/PKA and arachidonic acid signal transduction cascades. Increases in $[Ca^{2+}]_c$ have also been observed subsequent to DA D1 receptor activation (Chang et al., 2000). Furthermore the identification of multiple, pharmacologically distinct, intracellular Ca^{2+} pools in goldfish somatotropes suggests the possibility that in addition to selective involvement of

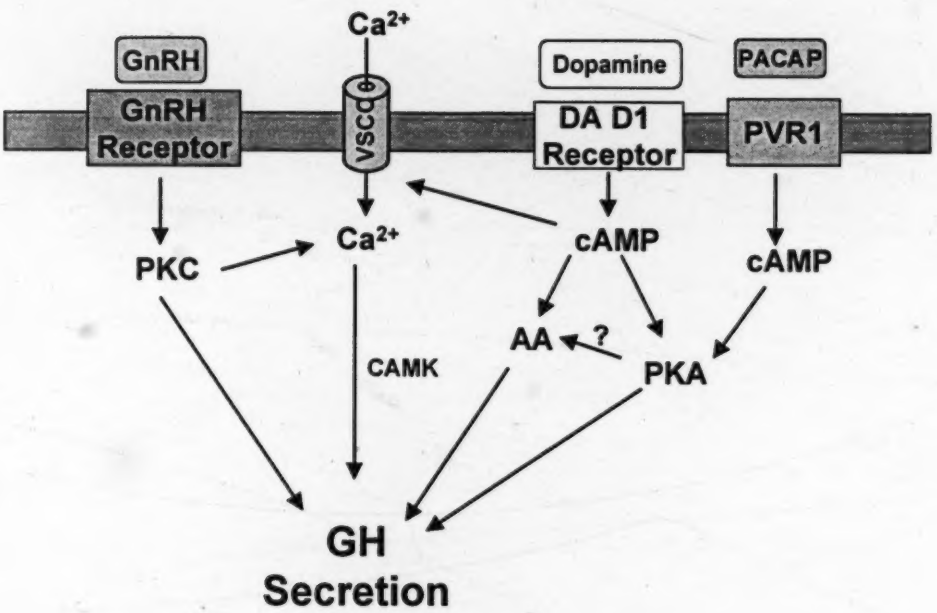


Figure 3. Representation of the known GH secretion pathways in response to GnRH, DA, and PACAP in goldfish somatotropes (modified from Chang et al., 2000).

PKC and PKA, the mechanisms and sites of Ca^{2+} mobilization may also participate in differential regulation of GH secretion by DA and both sGnRH and cGnRH-II (Johnson, 2000; Chang et al., 2000).

In addition to DA and GnRH, PACAP, GHRH, and neuropeptide Y are other neuroendocrine factors that have been identified to play a role in the direct stimulatory control of GH secretion in the goldfish pituitary. Recently, the involvement of the cAMP/PKA pathway in PACAP stimulation of GH secretion has also been established (Wirachowski et al., 2000; Wong et al., 1998).

The primary inhibitory control of GH release in the goldfish pituitary is through the actions of SRIH. SRIH has been shown to work directly at the level of the somatotrope, and at intracellular target sites distal to cAMP production, PKC activation, and increases in $[Ca^{2+}]_c$ to inhibit stimulated GH release (Yunker and Chang, 2001; Kwong and Chang, 1997). Furthermore, three isoforms of SRIH have been identified in the goldfish brain and have been proposed to affect GH secretion differentially (Yunker et al., 2001). Two other neuroendocrine factors, serotonin and norepenephrine, have also been demonstrated to act on pituitary cells to inhibit GH secretion in the goldfish (Peng and Peter, 1997). Like SRIH, norepinephrine has been shown to inhibit stimulated GH release by actions subsequent to cAMP production, PKC activation and increases in $[Ca^{2+}]_c$ (Yunker et al., 2000).

1.9 Goals and objectives

The highly reactive and unstable nature of NO has made it a notoriously difficult, yet extremely interesting molecule to study. These properties, along with the fact that NO seems to be involved in the regulation of diverse functions in nearly every cellular system, have led to the many controversies surrounding NO's specific actions in different cells and tissues. The goldfish pituitary is one of the most extensively characterized systems in terms of the multi-factorial control of hormone secretion and signal transduction cascades. The relative importance of Ca^{2+} in mediating the hormone-releasing regulatory actions of major neuroendocrine factors in goldfish predicts a possible participation of the NOS/NO system in the control of GH secretion. In addition, this model allows for the identification of single pituitary cell types amongst either a fixed or live, mixed pituitary cell population based solely on morphological criteria that are identifiable with simple DIC optics (Van Goor et al., 1994). This property allows for single cell experimentation without long cumbersome separation techniques or other chemical treatments for identification of cell types. All these characteristics make the goldfish an exceptional model to investigate the role of such a controversial and technically problematic molecule.

In this thesis I have set out to achieve four main goals. One of these goals is to establish if NO can be produced in pituitary cells of goldfish. This possibility is investigated by immunocytochemistry in conjunction with the unique cell identification technique for goldfish pituitary cells, Western blotting and by measurements of the formation of NO and its byproducts. A second goal is to

further understand the control of GH secretion in the goldfish by establishing if NO is involved in the regulation of GH secretion in this model system. Another goal is to begin to elucidate the signal transduction pathway used by NO within goldfish pituitary cells. The final goal is to determine if NO is part of the signal transduction pathways of some of the known regulators of GH secretion. These last three objectives are investigated using pharmacological manipulations of goldfish pituitary cells in primary culture in either prolonged or time course experiments, followed by the measurement of hormone release and/or cGMP production. By setting out to investigate the role of NO in this well characterized system it may be possible to shed some light on the biology of NO in general and to establish a new framework under which the biology of NO in other systems can be understood. In addition, this work will fill in a few blanks, and add some more questions to the complexity of the GH-release control system in the goldfish.

2. Materials and Methods

2.1 Animals

All animal care and experimental protocols used are approved by the animal care committee of the University of Alberta in accordance with national guidelines. Common goldfish (*Carassius auratus*; 8-13 cm in body length) are purchased from Grassyforks Fisheries (Martinsville, IN, USA), Ozark Fisheries (Stoutland, MO, USA), or Mount Parnel Fisheries (Mercersburg, PA, USA) and held in flow-through aquaria (1800 L) immediately upon arrival. Fish are maintained at 17-20°C on a simulated photoperiod (adjusted weekly to the times of sunrise and sunset in Edmonton, AB, Canada) and fed to satiation with commercial fish food daily. Fish are acclimated to the above conditions for at least 7 days prior to use and are usually used within 30 days of arrival. For these studies, post-pubertal male and female fish from all stages of the gonadal recrudescence/maturation cycle were used.

2.2 Drugs

Stock solutions of sGnRH, cGnRH-II (two endogenous GnRH forms in goldfish; Peter and Chang, 1999), mammalian $SRIH_{14}$ (one of the endogenous forms in goldfish; Kwong and Chang, 1997), PACAP-38 (mammalian $PACAP_{1-38}$; Peninsula Laboratories, Belmont, CA), sodium nitroprusside (SNP), aminoguanidine hemisulfate (AGH), and PTIO (2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; Calbiochem, La Jolla, CA) were prepared with distilled deionized water. SKF38393 ((±)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-

benzazepine-7,8-diol hydrochloride), S-Nitroso-N-acetylpenicillamine (SNAP), 7-nitroindazole (7-NI), rutin hydrate, ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), 1400W (N-(3-Aminomethyl) benzylacetamidine, dihydrochloride; Calbiochem), and dibutyryl guanosine 3':5'-cyclic monophosphate (dbcGMP; Sigma, Oakville, ON, Canada), were dissolved in dimethyl sulfoxide (DMSO). LY 83583 (6-Anilino-5,8-quinolinequinone; Calbiochem) was dissolved in ethanol. Aliquots of stock solutions were stored at -20°C until use, when they were diluted to final concentrations in testing medium. The highest concentration of DMSO was <0.1% which has no effect on basal hormone release or cell membrane ion currents (Wong et al., 1992). Rabbit antibody against rat bNOS, (fragment 251-270; Research Biochemicals International) and mouse iNOS (fragment 1131-1144; Calbiochem) were stored in aliquots at -20°C until use, when they were diluted to their final concentrations. Stock solution of goat anti-rabbit IgG TRITC-conjugate (Sigma) was stored at 5°C until use, when it was diluted to its final concentration.

2.3 Pituitary cell dispersion

Fish were anesthetized in 0.05% tricane methanesulfonate (Syndel, Vancouver, BC, Canada) prior to decapitation. Pituitaries from both male and female goldfish were removed and their cells were dispersed using a controlled trypsin/DNase treatment procedure (Chang et al., 1990). Pituitary cells were then cultured overnight and used for either immunocytochemical studies,

hormone release experiments, nitrite assay, cGMP assay, or DAF-2 imaging (see Appendix A).

2.4 Immunocytochemistry

Dispersed pituitary cells were cultured overnight in poly-L-lysine coated glass-bottom petri dishes containing plating medium (Medium 199 with Earl's salts, 25 mM HEPES, 2.2 g/L sodium bicarbonate, 100 000 U/L penicillin, 100 mg/L streptomycin, and 1% horse serum, pH 7.18; Gibco, Grand Island, NY, USA) under 5% CO_2, in saturated humidity, and at 28°C as previously described (Van Goor et al., 1994). Dispersed cells were fixed in Zamboni's 2X fixative (4% paraformaldehyde and 7.5% saturated picric acid in Dulbecco's Ca^{2+}-free phosphate buffered saline [0.2 g/L $NaH_2PO_4 \cdot H_2O$, 2.6 g/L $Na_2HPO_4 \cdot H_2O$, and 8.8 g/L NaCl]; pH 7.3) for 60 min. After fixation, the cells were washed, 3 X 10 min, with Dulbecco's Ca^{2+}-containing phosphate buffered saline (1 X D-PBS [0.1 g/L $CaCl_2$, 0.2 g/L KCl, 0.2 g/L KH_2PO_4 , 0.1 g/L $MgCl_2 \cdot 6H_2O$, 8.0 g/L NaCl, and 2.16 g/L $Na_2HPO_4 \cdot 7H_2O$]). Cells were incubated in blocking solution (1 X D-PBS containing 0.4% Triton X-100 (Sigma) and 10% horse serum (Gibco)) for 30 min prior to treatment with primary antibody. Cells were incubated overnight with primary antisera (rabbit anti-bNOS or rabbit anti-iNOS; diluted 1:500 in 1 X D-PBS containing 0.4% Triton X-100 and 0.2% horse serum). Cells were then washed, 4 X 7 min, with 1 X D-PBS containing 0.4% Triton X-100 and then incubated in goat anti-rabbit IgG conjugated to rhodamine (diluted 1:400 in 1 X D-PBS containing 0.4% Triton X-100 and 0.2% horse serum) for 60 min.

Following this incubation, cells were washed (4 X 5 min) with 1 X D-PBS containing 0.4% Triton X-100 and then with 1 X D-PBS (2 X 10 min) before mounting in either glycerol or methyl salicylate (Sigma). All treatments were carried out at room temperature except for the fixation and primary antisera incubations, which were carried out at 4°C. Cells were viewed and photographed using a Nikon Diaphot TMD inverted microscope equipped with epifluorescence illumination and Nomarski differential interference-contrast (DIC) optics. To facilitate the localization of bNOS and iNOS, pituitary GH cells were identified morphologically using previously described criteria (Van Goor et al., 1994). Briefly, GH cells can be recognized, with greater than 96% accuracy, by their distinctive ovoid nucleus, centrally located nucleolus, and cytoplasmic extensions using DIC optics (Fig. 4). These features can all be appreciated by focusing through all planes of the image. Cells identified by this method have been shown to respond to known neuroendocrine stimulators of GH release with increases in intracellular calcium signals in single-cell Fura-2 imaging studies (Johnson et al., 2000).

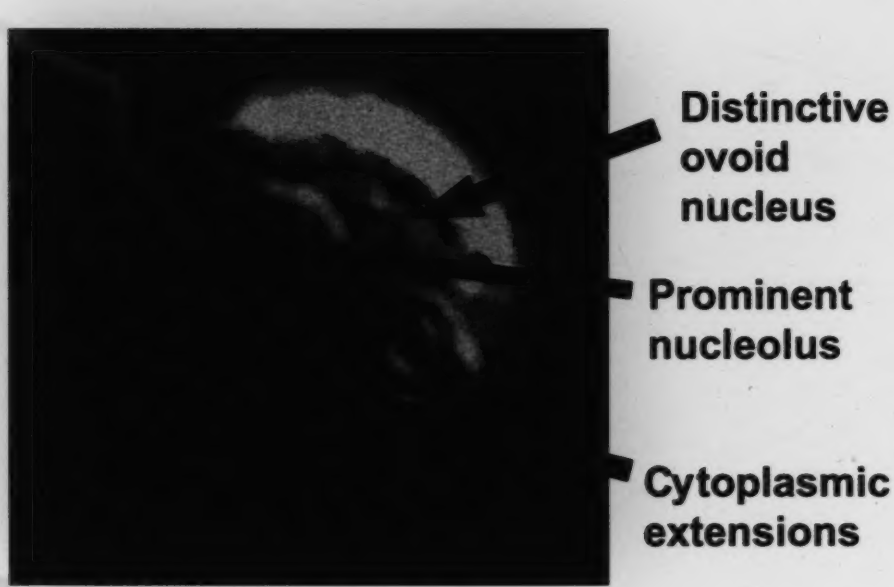


Figure 4. A goldfish somatotrope viewed using DIC optics. Arrows point to the three major identifying features visible with DIC optics.

2.5 Static incubation hormone release studies

Dispersed pituitary cells were cultured overnight in plating medium in 24-well-culture plates (0.25 X 10^6 cells/ml/well) under 5% CO_2, in saturated humidity at 28°C as previously described (Chang et al., 1990). Prior to experiments, cells were washed with testing media (Medium 199 with Hanks' salts, 25 mM HEPES, 2.2 g/L $NaHCO_3$, 0.1% bovine serum albumin, 100 000 U/L penicillin, and 100 mg/L streptomycin, pH adjusted to 7.18 with NaOH; Gibco) and allowed to rest for 1 hour under the above conditions. Cells were then incubated under similar conditions in testing medium supplemented with various secretagogues and inhibitors for 2 hour, after which the medium was removed and stored at -20°C. Inhibitors were usually added 10 minutes prior to addition of secretagogues. GH content was measured by a previously validated radioimmunoassay (Marchant et al., 1987). All treatments were tested in either triplicate, quadruplicate, or sextuplicate, and each experiment was performed a minimum of three times. Results from replicate experiments were expressed as a percentage of basal release (unstimulated control) and pooled data (mean ± SEM) are presented. Statistical analyses were performed using analysis of variance (ANOVA) followed by Fisher's protected least significant difference (LSD) test. Differences between groups were considered significant when $P<0.05$.

2.6 Perifusion studies on hormone release

For the analysis of acute hormone release responses to various stimulators and inhibitors, cell column perifusion studies were performed as

previously described (Wong et al., 1992). Dispersed goldfish pituitary cells were cultured for 16-40 hr on preswollen Cytodex-I beads in plating media supplemented with 1% horse serum, under 5% CO_2, saturated humidity, and 28°C. Prior to experimentation, cells were loaded onto perifusion columns (2 x 10^6 cells/column) and perifused with testing media for 4 hr at a rate of 15 ml/hr, after which a stable basal secretion rate was usually established (Wong et al., 1992). Experiments began with collection of 5-min fractions of perifusate and, in some instances, changed to 1-min fractions to increase the resolution of the GH response. Secretagogues were usually applied as a 5-min pulse while treatments with inhibitors generally commenced 10-min prior to secretagogue addition. GH content of perifusates was determined by radioimmunoassay. Values are normalized to the mean of the four measurements immediately prior to the first treatment (% pretreatment). This transformation has been previously described and it has been demonstrated that this normalization is required to pool results from independent columns in our in vitro system (Wong et al., 1992). Total GH response was quantified by integrating the "area under the curve" for the duration of the response to stimulatory treatment, which was typically 20-30 min. In most instances, representative GH release traces were shown for clarity, while "net response" data from pooled results were always presented. Statistical analysis was performed using a *t*-test in cases of only two variables or ANOVA followed by protected Fisher's least significant difference post-hoc test in the case of multiple variables. Where large variance existed between groups, a non-

parametric test (Kruskal-Wallis followed by Mann-Whitney U-test) was used. Differences were considered significant when $P < 0.05$.

2.7 Cyclic GMP assay

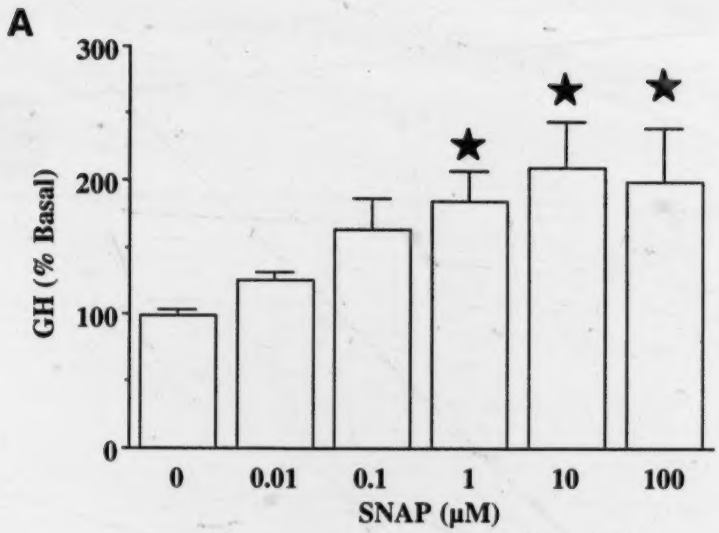
Intracellular and released cGMP levels were analyzed by enzyme immunoassay (EIA). 50 µl of samples were used following the standard static incubation experiments described in section 2.5 except that phenyl red-free media was used for washing and static incubation experiment. All chemicals and products were supplied in the cGMP EIA kit and all treatments of samples performed according to instructions provided by the manufacturer (Cayman chemical, Ann-Arbor, MI). For estimation of cGMP content, cells were lysed in double distilled deionized water following removal of media at the end of secretion testing and sonication for 10 min. Samples were run on 96-well assay plates and absorbance was measured at 405 nm following the procedures from the kit. Cyclic GMP concentration was determined by interpolation using a standard curve prepared on each plate.

3. Results

3.1 NO effects on basal growth hormone secretion

To examine if activation of NO pathways affects GH release from the goldfish pituitary, two NO donor molecules, SNAP and SNP (Oh and McCaslin, 1995), were applied in varying concentrations (0.01-100 μM) to goldfish pituitary cells in static culture. Both SNAP and SNP increased GH release in a dose-dependent manner. Compared to non-stimulated controls, significant increases in GH were observed after addition of 1-100 μM SNAP (Fig. 5A) and subsequent to addition of 10 and 100 μM of SNP (Fig. 5B). To further characterize this release response, SNP (100 μM) and light-inactivated SNAP (10 μM) were given in a 5-min pulse to primary cultures of goldfish pituitary cells in cell column perifusion experiments. The light-inactivated SNAP did not elicit a significant GH release response, while SNP induced an acute, robust, but somewhat variable response (Fig. 6). The GH release response to SNP lasted approximately 15 min before returning to basal levels. To ensure that the cells had remained alive and reactive during the entire course of the experiment, they were stimulated with SKF 38393, a DA D1 receptor agonist, which is a well-documented GH secretagogue (Peter and Chang, 1999) prior to and following the addition of SNP or inactivated SNAP. The cells reacted as expected to SKF 38393 both before and after challenges with the NO donor molecules. The slight diminishing of the GH response to the second pulse of SKF 38393 has been previously observed

Figure 5. NO stimulates GH secretion in 2-hour static culture. GH release response to varying concentrations of SNAP (A; 0.01-100 μM) and SNP (B; 0.01-100 μM). Results are presented as mean ± SEM (% basal GH). Star identifies significant differences from control ($P<0.05$).


A
300
200
100
0
GH (% Basal)
0
0.01
0.1
1
10
100
SNAP (μM)

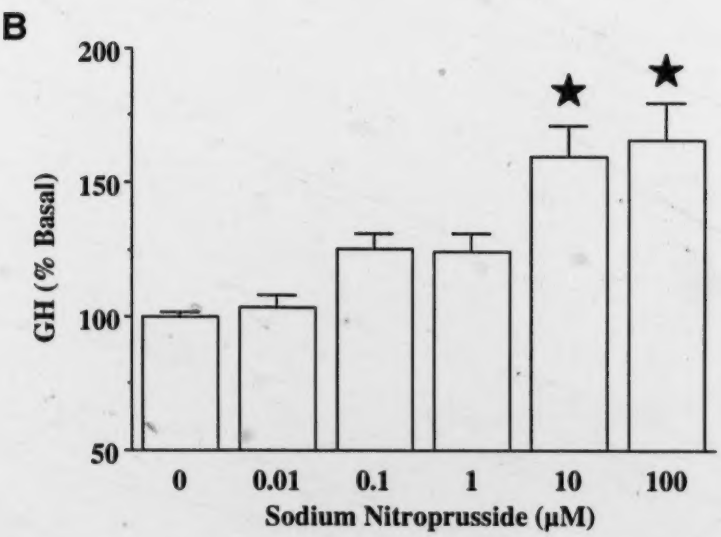
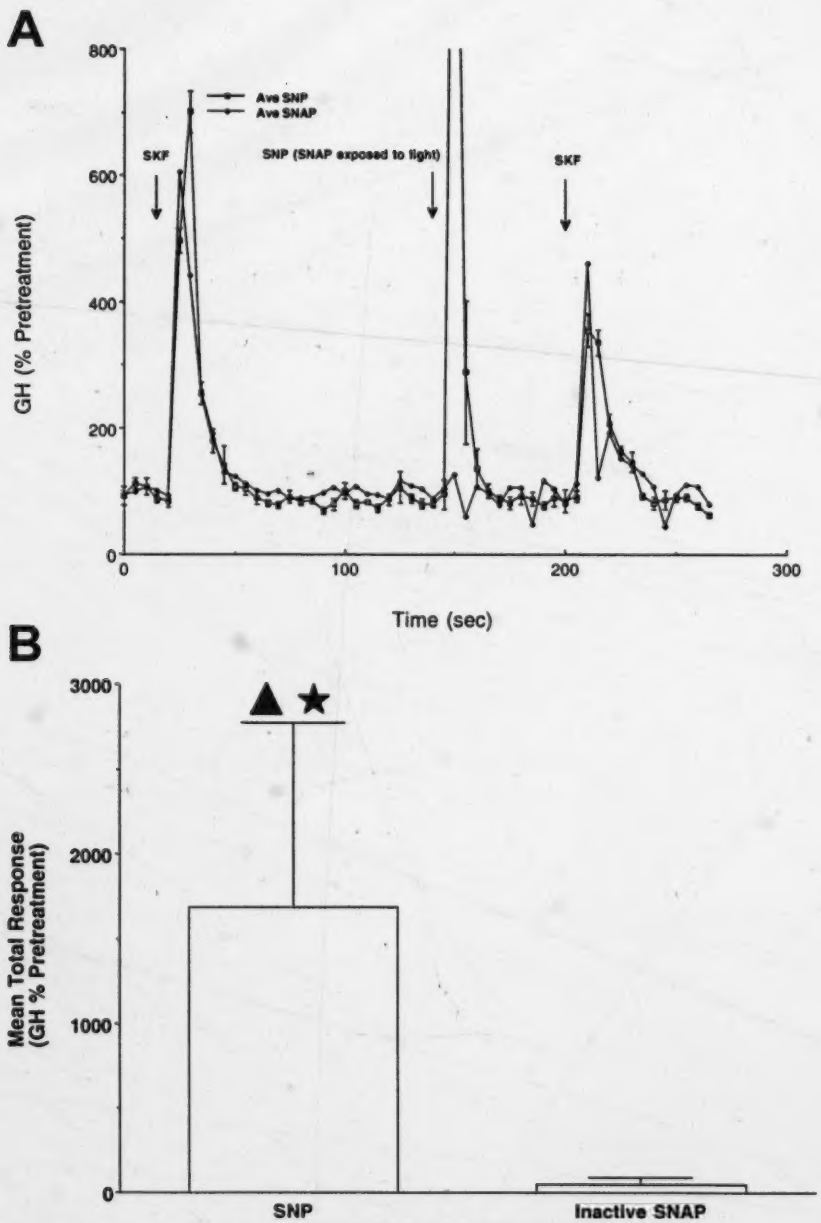

B
200
150
100
50
GH (% Basal)
0
0.01
0.1
1
10
100
Sodium Nitroprusside (μM)

Figure 6. NO stimulates GH secretion in cell column perifusion. **A.** Temporal profile of the GH release response to SNP (100 μM) or light-inactivated SNAP (10 μM) and SKF 38393 (1 μM; SKF). Arrows indicate 5-min applications of various secretagogues. **B.** Total response to SNP and light-inactivated SNAP. Results are presented as mean ± SEM (% pretreatment). Star indicates significant difference from light-inactivated SNAP ($P<0.05$). Triangle indicates significant difference from "zero response".

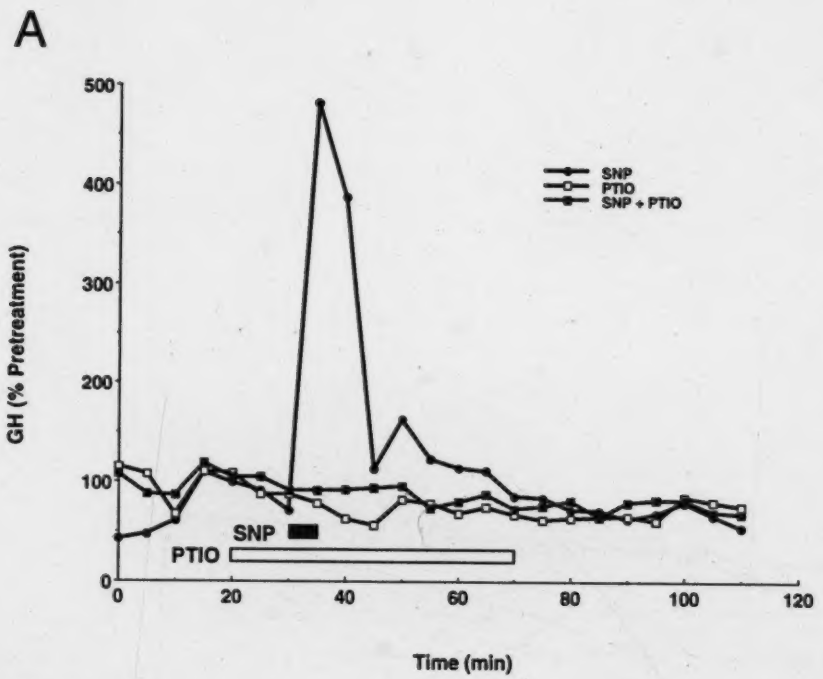

A
800
600
400
200
0
Ave SNP
Ave SNAP
SKF
SNP (SNAP exposed to light)
SKF
GH (% Pretreatment)
0
100
200
300
Time (sec)
B
3000
2000
1000
0
Mean Total Response (GH % Pretreatment)
SNP
Inactive SNAP

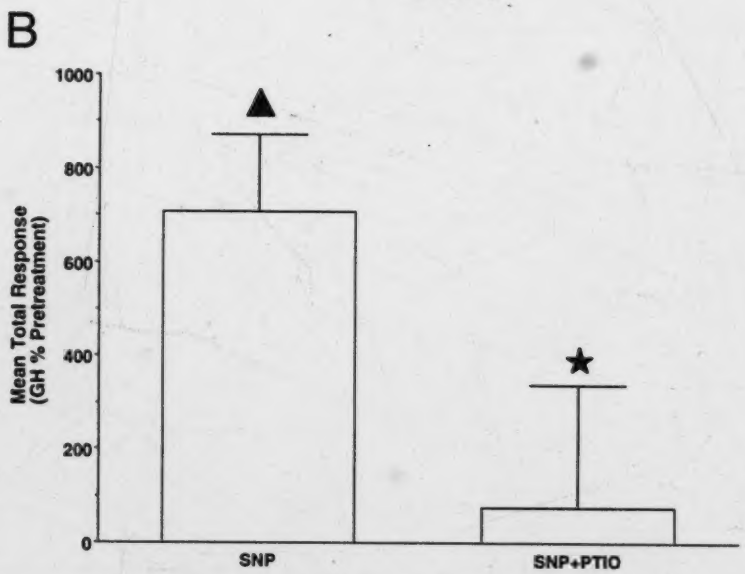
and can be attributed to homologous desensitization. Importantly, the GH responses to SKF 38393 following treatments with the active and inactive NO donors were similar, suggesting that exposure to NO did not affect cell viability. That a GH response to the second SKF 38393 challenge was observed, also argues against cell death as a cause of the lack of a GH release response to the inactivated SNAP. These data suggest that increased NO formation leads to an elevation of GH release from goldfish somatotropes.

To investigate whether the response to the NO donor molecules was indeed due to the spontaneous release of NO from these solutions and not from byproducts of NO formation or breakdown, the effects of SNP (100 μM) on GH release were tested both in the presence and absence of a molecule that scavenges NO in solution. Treatment with the NO scavenger PTIO (10 μM; Hogg et al., 1995) was carried out. PTIO application was commenced 10 min prior to a 5-min pulse of SNP and lasted for a total of 55 minutes. GH secretion was not stimulated by SNP in the presence of PTIO; in contrast, treatment with SNP alone stimulated a robust GH release response (Fig. 7). These data further support the hypothesis that NO is the effector molecule stimulating GH release from goldfish pituitary cells.

To examine if the NO-stimulated GH release could be reversed with a known GH release inhibitor, SRIH was applied to static cultures of goldfish pituitary cells along with SNP. Application of SRIH (100 nM) suppressed basal GH release and abolished the GH response to 100 mM SNP. SRIH significantly

Figure 7. NO stimulation of GH secretion is abolished by the use of a NO scavenger in cell column perifusion. **A.** Representative temporal profiles of SNP (100 μM)-stimulated GH secretion in the presence or absence of the NO scavenger PTIO (10 μM). Duration of treatments are indicated by the horizontal bars. **B.** Total response to SNP in the presence and absence of PTIO. Results presented as mean ± SEM (% pretreatment). Star indicates significant difference ($P < 0.05$) from the response to SNP alone. Triangle indicates significant difference from "zero response".


A
500
400
300
200
100
0
GH (% Pretreatment)
SNP
PTIO
SNP + PTIO
SNP
PTIO
0
20
40
60
80
100
120
Time (min)


B
1000
800
600
400
200
0
Mean Total Response
(GH % Pretreatment)
SNP
SNP+PTIO

decreased the SNP-stimulated GH response to levels observed in the presence of SRIH alone (Fig. 8). These data suggest that NO stimulation can be controlled by an inhibitory neuroendocrine regulator of GH secretion in goldfish.

To determine if cGMP, a signaling molecule known to mediate NO effects in many systems (McDonald and Murad, 1996), can also stimulate GH release in goldfish, the membrane-permeant cGMP analogue, dbcGMP, was applied to dispersed goldfish pituitary cells in static culture. Addition of 1 and 5 mM dbcGMP both significantly increased GH release to levels observed in response to a maximal stimulatory dose of 0.1 μM cGnRH-II, a well-documented endogenous stimulator of GH in goldfish (Fig. 9; Chang et al., 1990). These data suggest that cGMP mimics the ability of NO to stimulate GH secretion in goldfish pituitary cells. In addition, these data suggest the possibility that cGMP could be a mediator of the NO stimulatory pathway in these cells. To assess this possibility, the ability of SNP to stimulate GH release in the presence of an inhibitor of sGC (1 μM ODQ; Moro et al., 1996) was examined. In the presence of ODQ, cells did not respond to SNP with an increase in GH release (Fig. 10). Preliminary studies measured increases in cGMP formation subsequent to 2-hour static incubation with SNP (100 μM; from undetectable levels to between 0.2-0.3 pmol/L). These results, in conjunction with the observed ability of dbcGMP to increase GH release, strongly suggest that the NO stimulatory effect on somatotropes is mediated by activation of sGC and subsequent cGMP formation.

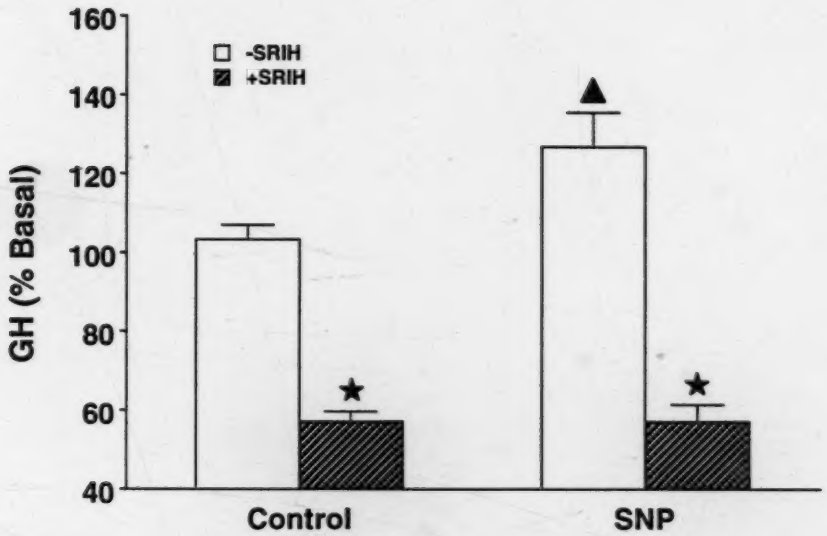


Figure 8. SNP (100 μM) stimulation of GH secretion is abolished in the presence of SRIH (100 nM) in 2-hour static incubation. Results are presented as mean ± SEM (% basal GH). Star indicates significance from non-SRIH treated group ($P < 0.05$). Triangle indicates significant difference from the corresponding control.

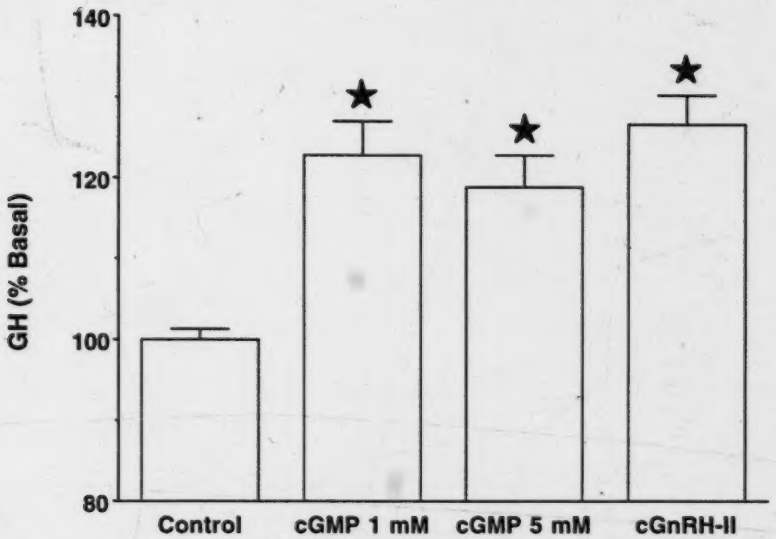
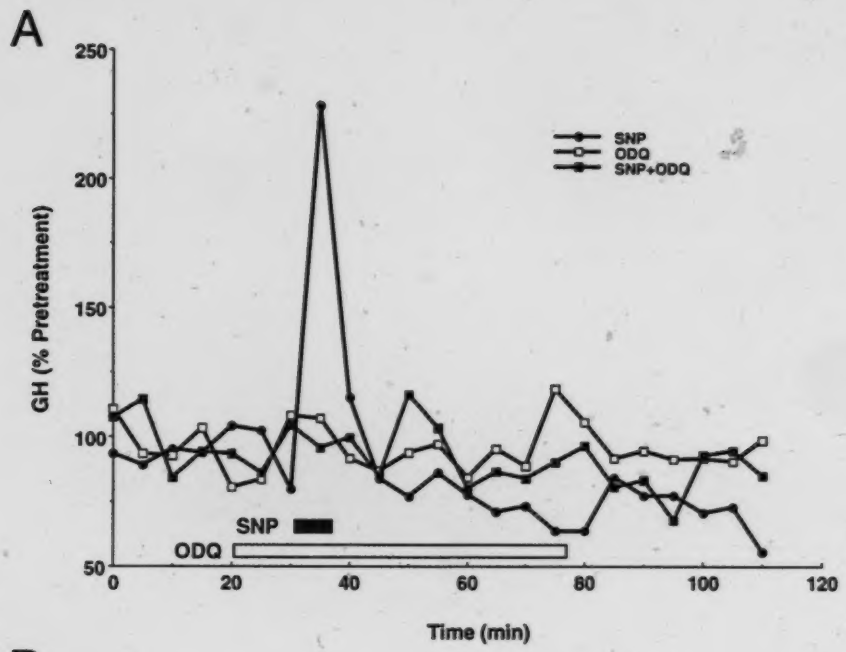
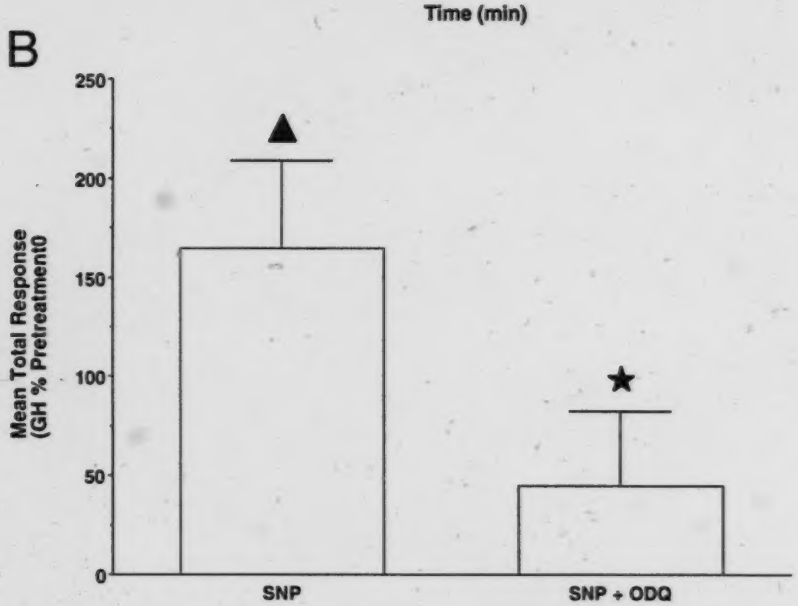


Figure 9. cGMP can stimulate GH secretion in 2-hour static culture. GH responses to 1 and 5 mM dbcGMP and to cGnRH-II (0.1 μM). Results are presented as mean ± SEM (% basal GH). Star indicates significant difference from control ($P < 0.05$).

Figure 10. sGC activity is required for NO stimulation of GH secretion in cell column perifusion. **A.** Representative temporal profiles of the GH release response to SNP (100 μM) in the presence or absence of ODQ (0.1 μM). Duration of treatments are indicated by the horizontal bars. **B.** Total response to SNP in the presence and absence of ODQ. Results presented as mean ± SEM (% pretreatment). Star indicates significant difference from response to SNP alone ($P<0.05$). Triangle indicates significant difference from "zero response".


A
250
200
150
100
50
GH (% Pretreatment)
SNP
ODQ
SNP+ODQ
SNP
ODQ
0
20
40
60
80
100
120
Time (min)


B
250
200
150
100
50
0
Mean Total Response
(GH % Pretreatment0
SNP
SNP + ODQ

3.2 NO mediation of GnRH-stimulated growth hormone release

While the preceding data shows that NO can stimulate the secretion of GH from cultured pituitary cells, it does not demonstrate a physiological role for NO in the regulation of GH secretion. Although there remains a distinct possibility that NO itself acts as a neuroendocrine signaling molecule in this system, it is also possible that NO acts as a second messenger molecule, transducing the signal of an already documented stimulator of GH release. As discussed in section 1.8, there are two endogenous GnRH isoforms in the goldfish and both are known to stimulate GH secretion. While much is known about the signal transduction cascades induced by both sGnRH and cGnRH-II (for a review see Chang et al, 2000), there is still more that remains unknown. Therefore, it is possible that NO could be one of the "missing links" that is generated intracellularly between the time of GnRH binding to its cell surface receptor and the exocytosis of GH.

3.2.1 Effects of NOS inhibitors on GnRH-stimulated growth hormone release

To begin investigating whether NO is involved in the GH release response of goldfish somatotropes to the two endogenous GnRH isoforms, 2-hour static incubation experiments were carried out. In order to block the production of NO, an inhibitor of the NOS enzyme was added prior to stimulation with either sGnRH or cGnRH-II. The first NOS inhibitor tested, 7-nitroindazole (7-NI), was one specific for the mammalian brain isoform of NOS. This inhibitor was chosen because the anterior pituitary (adenohypophysis), that contains somatotropes, is anatomically located in the vicinity of the brain, is directly innervated by

hypothalamic neurons, and is at least in part from neural origin (Gorbman, 1995). 7-NI has been shown to inhibit bNOS with a K_i of approximately 700 nM (Klatt et al., 1994). Pretreatment of cells in static culture with 1 μM 7-NI did not alter the GH release responses to previously demonstrated, maximal stimulatory concentrations of cGnRH-II (0.1 μM) and sGnRH (0.1 μM; Chang et al., 1990; Fig. 10). In further experiments, two other doses of 7-NI (10 and 0.1 μM) also did not reduce cGnRH-II- or sGnRH-stimulated GH secretion (not shown). Furthermore, 7-NI did not significantly alter basal GH secretion (Fig. 11). These data imply that NO formation via bNOS activity may not play a role in the regulation of GH release by either of the two endogenous GnRHs.

The isoform of NOS in the goldfish pituitary gland is currently unidentified and therefore may be sufficiently different from mammalian bNOS insomuch as not to be inhibited by 7-NI. It is also possible that the isoform present in goldfish pituitary cells may be more pharmacologically and/or structurally similar to either mammalian eNOS or iNOS. With these possibilities in mind, the effects of a broad spectrum NOS inhibitor, aminoguanidine hemisulfate (AGH; Holstad et al., 1997; Laszlo et al., 1995) was examined. Application of 1 mM AGH significantly decreased the GH release response to previously demonstrated maximal stimulatory doses of both cGnRH-II and sGnRH (0.1 μM; Chang et al., 1990; Fig. 12). This observation suggests that NO is involved in GnRH-induced GH release. However, AGH did not attenuate basal GH secretion (Fig. 12) suggesting that NO may only be involved in the regulated GH secretion pathway.

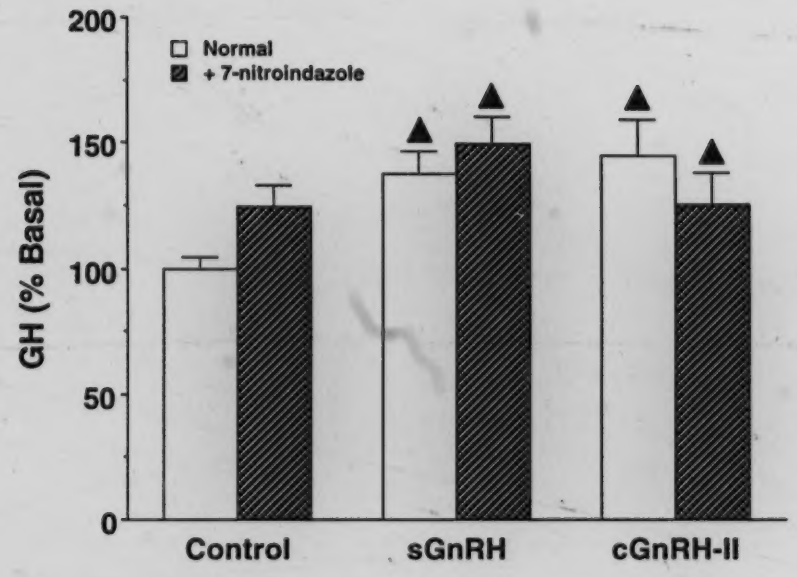


Figure 11. GH response to sGnRH and cGnRH-II (0.1 μM) is unaffected by a mammalian bNOS inhibitor (7-NI; 1 μM) in 2-hour static culture. Results are presented as mean ± SEM (% basal GH). Triangles indicate significant difference from control ($P<0.05$). No significant differences are observed between 7-NI-treated and non-treated groups.

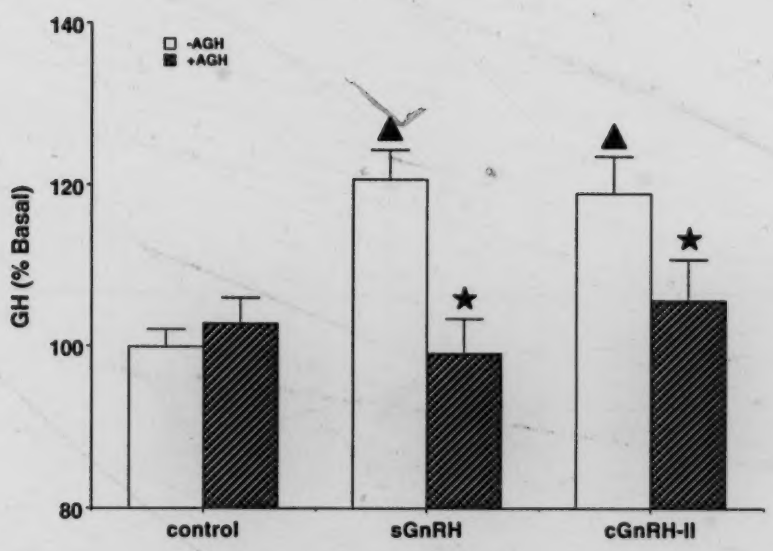


Figure 12. Inhibition of sGnRH and cGnRH-II (0.1 μM) stimulation of GH by AGH (1 mM) in 2-hour static culture. Results presented as mean ± SEM (% basal GH). Star indicates significant difference from non-AGH-treated groups ($P<0.05$). Triangle indicates significant difference from the corresponding control.

To further investigate the role that NO may play in mediating the GnRH response in pituitary cells and to attempt to narrow down the pharmacological characteristics of the isoform of NOS present in goldfish pituitary cells, a NOS inhibitor specific for mammalian iNOS (1400W) was used. 1400W has been reported to selectively inhibit iNOS with a K_i of approximately 10 nM (Garvey et al., 1997). Application of the selective iNOS inhibitor 1400W (1 μM) did not significantly alter basal secretion (Fig. 13). However, cGnRH-II-stimulated secretion was abolished in the presence of 1400W and sGnRH was unable to stimulate GH secretion in the presence of 1400W (Fig. 13). These data suggest that NO formation via an enzyme that is pharmacologically similar to a mammalian iNOS may play a role in the control of GH secretion stimulated by the two endogenous GnRHs. To characterize the GH release response in the presence of iNOS inhibition more specifically, the temporal characteristics were analyzed in cell column perifusion experiments. Cells were pretreated with 1400W (1 μM) for 10 min before receiving a 5-min pulse of cGnRH-II (0.1 μM). 1400W by itself had no significant effect on the GH release profile (Fig. 14A). cGnRH-II caused an increase in GH release of 15-20 min in duration, but in the presence of 1400W this release response to cGnRH-II was abolished Fig. 14B). These data further suggest a role for NO generation as a critical step in the cGnRH-II stimulation of GH release from somatotropes. The temporal characteristics of the effect of 1400W on sGnRH-stimulated GH release were also investigated to look for similarities with, and/or differences from, the effects

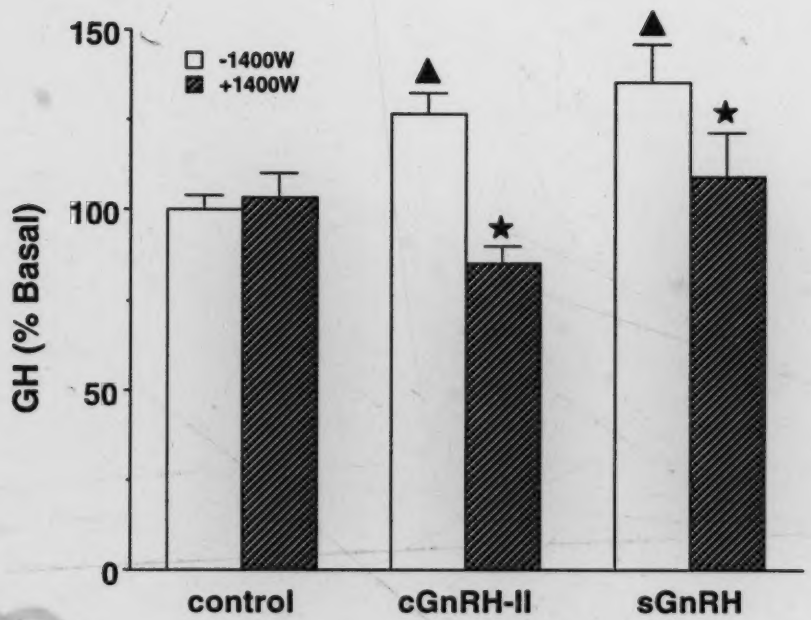
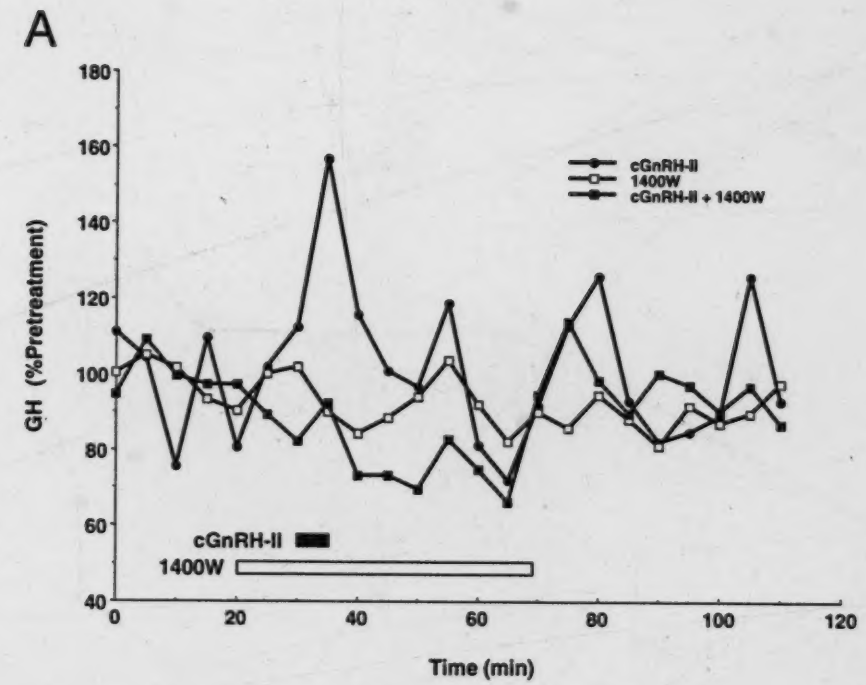
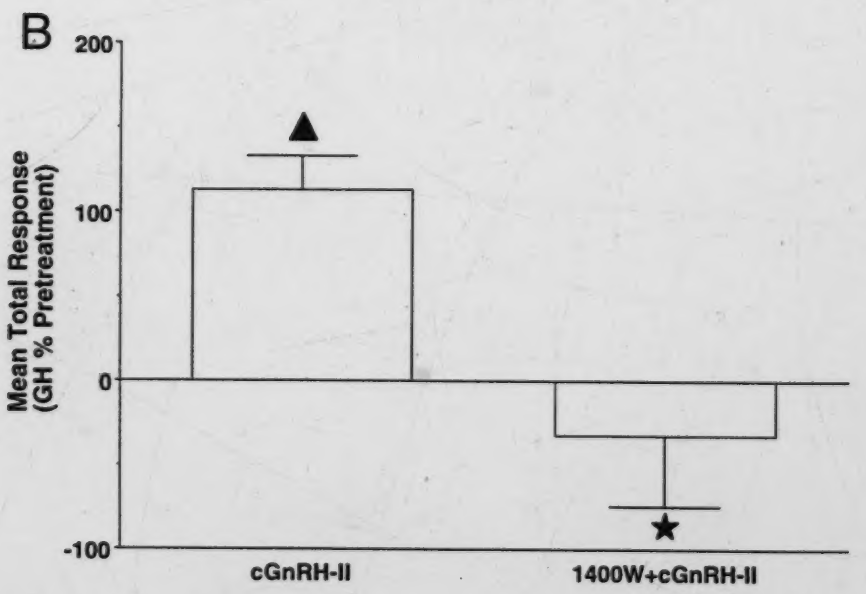


Figure 13. Inhibition of cGnRH-II and sGnRH (0.1 μM)-stimulated GH secretion by a mammalian iNOS inhibitor (1400W; 1 μM) in static culture. Results are presented as mean ± SEM (% basal GH). Star indicates significance from non-1400W-treated groups ($P<0.05$). Triangle indicates significant difference from the corresponding control.

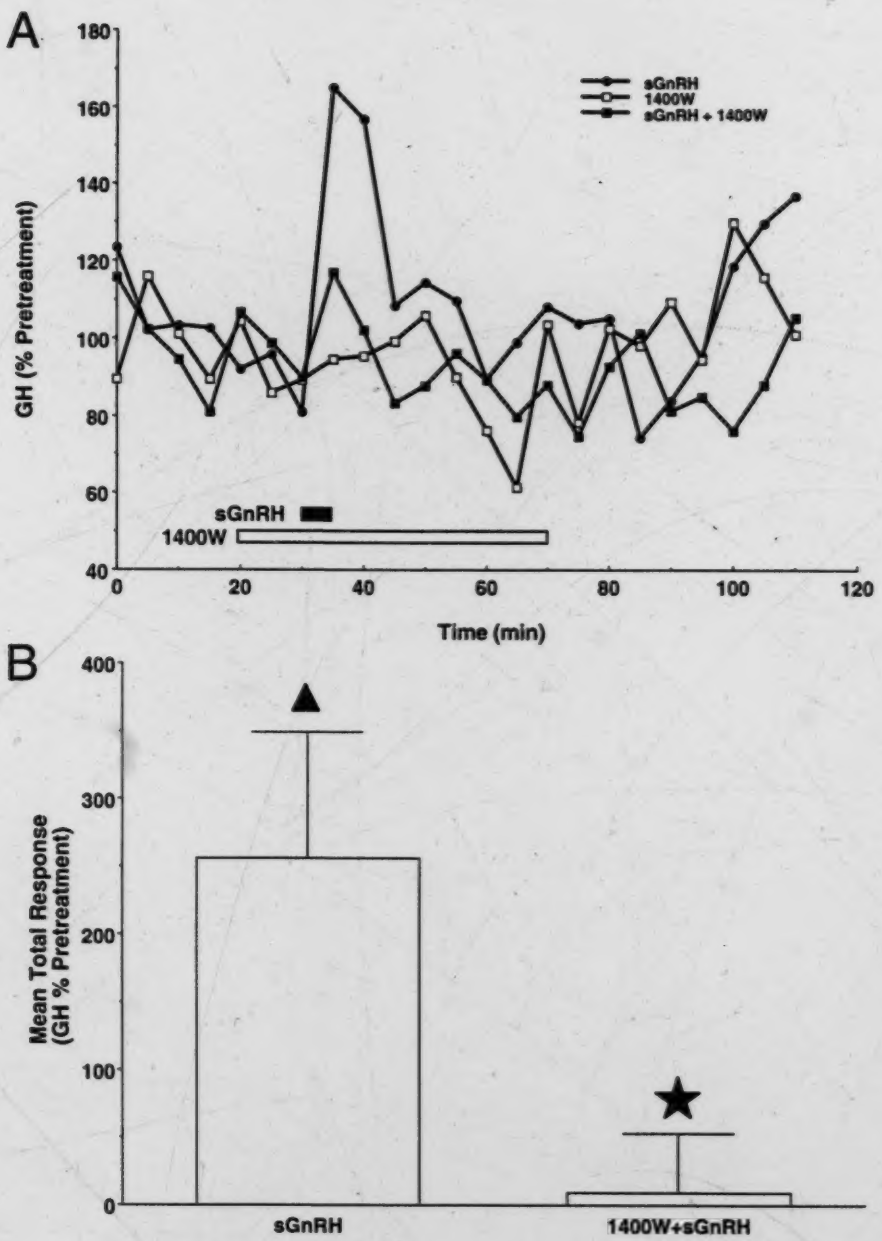
Figure 14. NOS activity is required for cGnRH-II stimulation of GH in cell column perifusion. **A.** Representative temporal profiles of GH release in response to cGnRH-II (0.1 μM) in the presence or absence of 1400W (1 μM). Duration of GnRH and inhibitor treatments are indicated by the horizontal bars. **B.** Total response to cGnRH-II in the presence and absence of 1400W. Results are expressed as mean ±SEM (% pretreatment). Star indicates significant difference from non-1400W-treated groups ($P<0.05$). Triangle indicates significant difference from "zero response".


A
180
160
140
120
100
80
60
40
GH (%Pretreatment)
cGnRH-II
1400W
cGnRH-II + 1400W
cGnRH-II
1400W
0
20
40
60
80
100
120
Time (min)


B
200
100
0
-100
Mean Total Response
(GH % Pretreatment)
cGnRH-II
1400W+cGnRH-II

on cGnRH-II-stimulated GH release. Cells were pretreated with 1400W (1 μM) for 10 min prior to stimulation with a 5-min pulse of sGnRH (0.1 μM). While treatment with sGnRH alone induced a spike of GH release lasting approximately 15 minutes, sGnRH was unable to induce significant GH release in the presence of 1400W (Fig. 15). These observations, in conjunction with the observations using cGnRH-II, strongly imply the involvement of NOS-like activity with sensitivity to iNOS inhibition in mediating the GH release response to both endogenous GnRHs in goldfish. Interestingly, the consistent inability of 1400W and AGH to alter basal GH release suggests that NOS activity may not be involved in the normal basal GH release regulation.

Figure 15. NOS activity is necessary for sGnRH stimulation of GH in cell column perifusion. **A.** Representative temporal profiles of the GH release response to sGnRH (0.1 μM) in the presence or absence of 1400W (1 μM). Duration of GnRH and inhibitor treatments are indicated by the horizontal bars. **B.** Total response to sGnRH in the presence and absence of 1400W. Results are expressed as mean ± SEM (% pretreatment). Star indicates significant difference from non-1400W-treated groups ($P<0.05$). Triangle indicates significant difference from "zero response".


A
180
160
140
120
100
80
60
40
GH (% Pretreatment)
sGnRH
1400W
sGnRH + 1400W
sGnRH
1400W
0
20
40
60
80
100
120
Time (min)
B
400
300
200
100
0
Mean Total Response
(GH % Pretreatment)
sGnRH
1400W+sGnRH

3.2.2 Effects of NO scavengers on GnRH-induced growth hormone release

To establish if the product of the NOS-like activity, which can be inhibited by 1400W and AGH as mentioned above, is actually NO, further experimentation was required. Cell permeable scavenger molecules known to soak up NO intracellularly and extracellularly were applied in cell column perifusion experiments with the two endogenous GnRHs. These molecules have extremely high affinity for NO and will "mop up" all NO in their presence; thus any NO generated, whether for action in an intracrine, autocrine, or paracrine fashion, will bind to these molecules and be unable to carry out its physiological function. Two such molecules are rutin hydrate and PTIO. Rutin hydrate performs this function in conjunction with its anti-oxidant properties that push the $NO^-/NO\bullet/NO^+$ equilibrium to the relatively un-reactive NO^-, as well as inhibiting NOS activity by inhibiting the oxidation of NADH to NAD^+ (Korkina and Afanas'ev, 1997). On the other hand, PTIO scavenges NO in a stoichiometric fashion without affecting NOS activity (Hogg et al., 1995).

Treatment with rutin hydrate (10 μM) alone had no significant effects on basal GH secretion (Fig. 16A). However, cells pretreated with rutin hydrate were unable to respond to 0.1 μM cGnRH-II with an increase in GH release (Fig. 16). Likewise, PTIO (10 μM) did not alter basal GH release, but attenuated the responses to a 5-min pulse of 0.1 μM sGnRH (Fig. 17). That PTIO has no anti-oxidant properties and was still able to suppress stimulated GH release, as in the case of rutin hydrate, reinforces the idea that scavenging NO (and not just inhibition of NOS activity) inhibits the stimulatory action of the two GnRHs on GH

Figure 16. Treatment with a NO scavenger inhibits cGnRH-II stimulation of GH in cell column perifusion. **A.** Representative temporal profiles of the GH release response to cGnRH-II (0.1 μM) in the presence or absence of rutin hydrate (10 μM). Duration of GnRH and scavenger treatments are indicated by the horizontal bars. **B.** Total response to cGnRH-II is abolished in the presence and absence of rutin hydrate. Results are expressed as mean ± SEM (% pretreatment). Star indicates significant difference from cGnRH-II alone ($P<0.05$). Triangle indicates significant difference from "zero response".

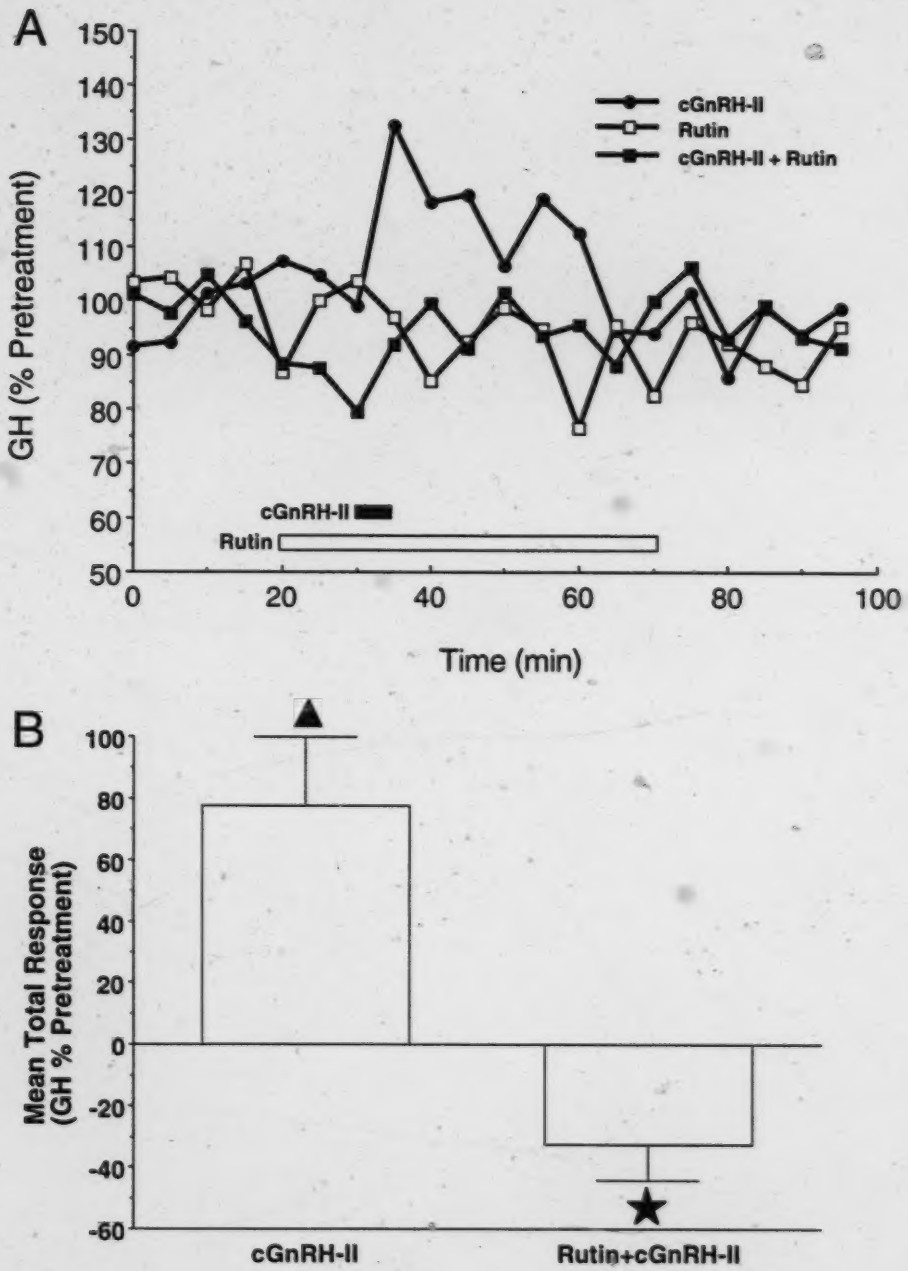
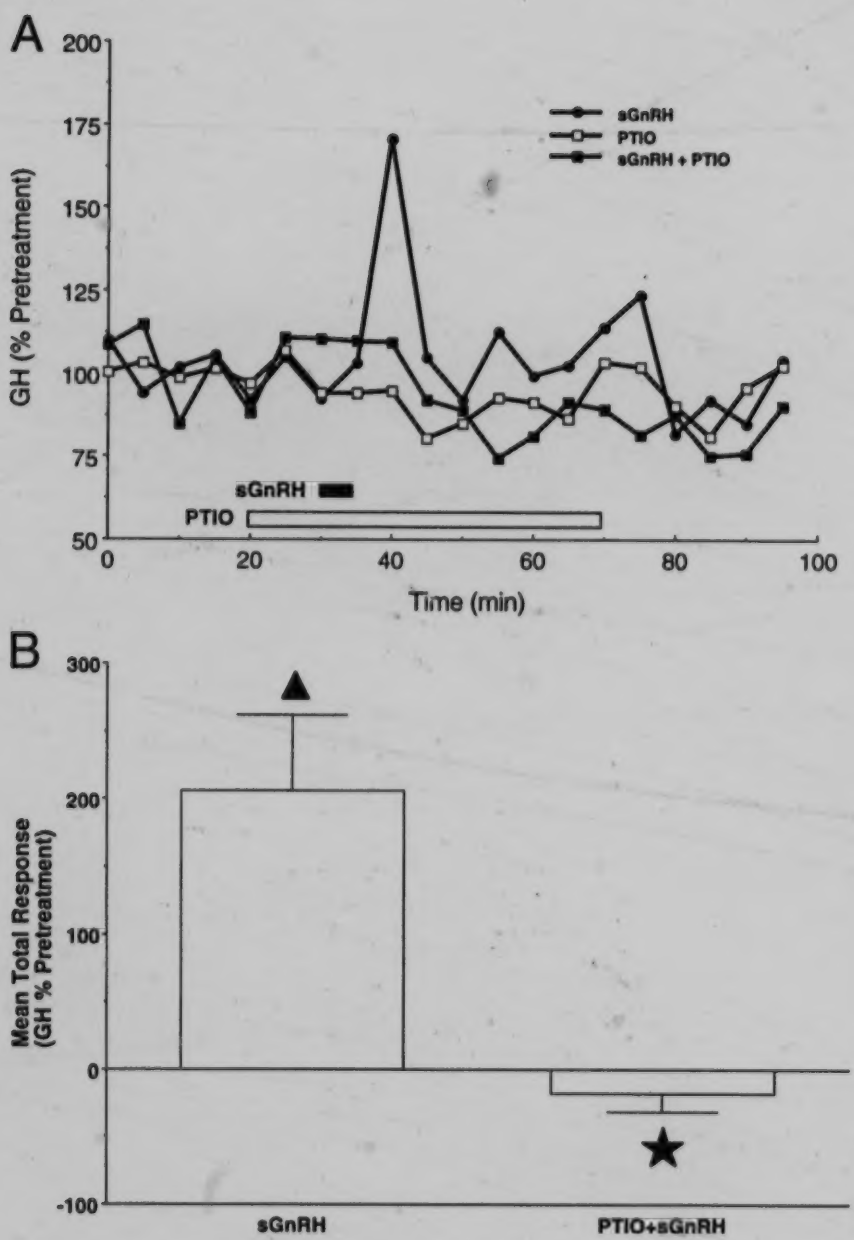

A
150
140
130
120
110
100
90
80
70
60
50
GH (% Pretreatment)
cGnRH-II
Rutin
cGnRH-II + Rutin
cGnRH-II
Rutin
0
20
40
60
80
100
Time (min)
B
100
80
60
40
20
0
-20
-40
-60
Mean Total Response
(GH % Pretreatment)
cGnRH-II
Rutin+cGnRH-II

Figure 17. Treatment with a NO scavenger inhibits sGnRH stimulation of GH in cell column perifusion. **A.** Representative temporal profiles of the GH release response to sGnRH (0.1 μM) in the presence or absence of PTIO (10 μM). Duration of GnRH and scavenger treatments are indicated by the horizontal bars. **B.** Total response to sGnRH in the presence and absence of PTIO. Results expressed as mean ± SEM (% pretreatment). Star indicates significant difference from sGnRH alone ($P<0.05$). Triangle indicates significant difference from "zero response".


A
200
175
150
125
100
75
50
GH (% Pretreatment)
sGnRH
PTIO
sGnRH + PTIO
sGnRH
PTIO
0
20
40
60
80
100
Time (min)
B
300
200
100
0
-100
Mean Total Response
(GH % Pretreatment)
sGnRH
PTIO+sGnRH

release. More importantly, these data further support the inference that NO is the affector molecule required for GH secretion induced by the GnRHs.

3.2.3 Possible involvement of sGC activity

The next step in characterizing the NO-mediated, GnRH-stimulated GH response is to characterize the second messenger system used by NO to increase GH release in response to GnRH stimulation. As previously described in section 3.1, NO actions on GH secretion appear to be mediated by activation of sGC and subsequent cGMP formation. To establish whether or not this same mechanism is involved in the apparent NO-mediated, GnRH-stimulated GH secretion, the sGC inhibitor ODQ was used in conjunction with the two endogenous GnRHs in cell column perifusion experiments. ODQ (0.1 μM) did not significantly alter basal GH secretion (Fig. 18A, 19A), suggesting that sGC is not involved in the constitutive GH release pathway. Stimulation with cGnRH-II alone produced the expected acute GH release response; however in the presence of ODQ, this response to cGnRH-II was abolished (Fig. 18). Likewise, exposure to ODQ blocked the GH release response to sGnRH (Fig. 19). These data suggest that NO, via the activation of the sGC/cGMP second messenger system, mediates cGnRH-II and sGnRH stimulation of GH release.

Figure 18. sGC activity is required for cGnRH-II stimulation of GH in cell column perifusion. **A.** Representative temporal profiles of the GH release response to cGnRH-II (0.1 μM) in the presence or absence of ODQ (0.1 μM). Duration of GnRH and inhibitor treatments are indicated by the horizontal bars. **B.** Total response to cGnRH-II in the presence and absence of ODQ. Results presented as mean ± SEM (% pretreatment). Star indicates significant difference from cGnRH-II alone ($P<0.05$). Triangle indicates significant difference from "zero response".

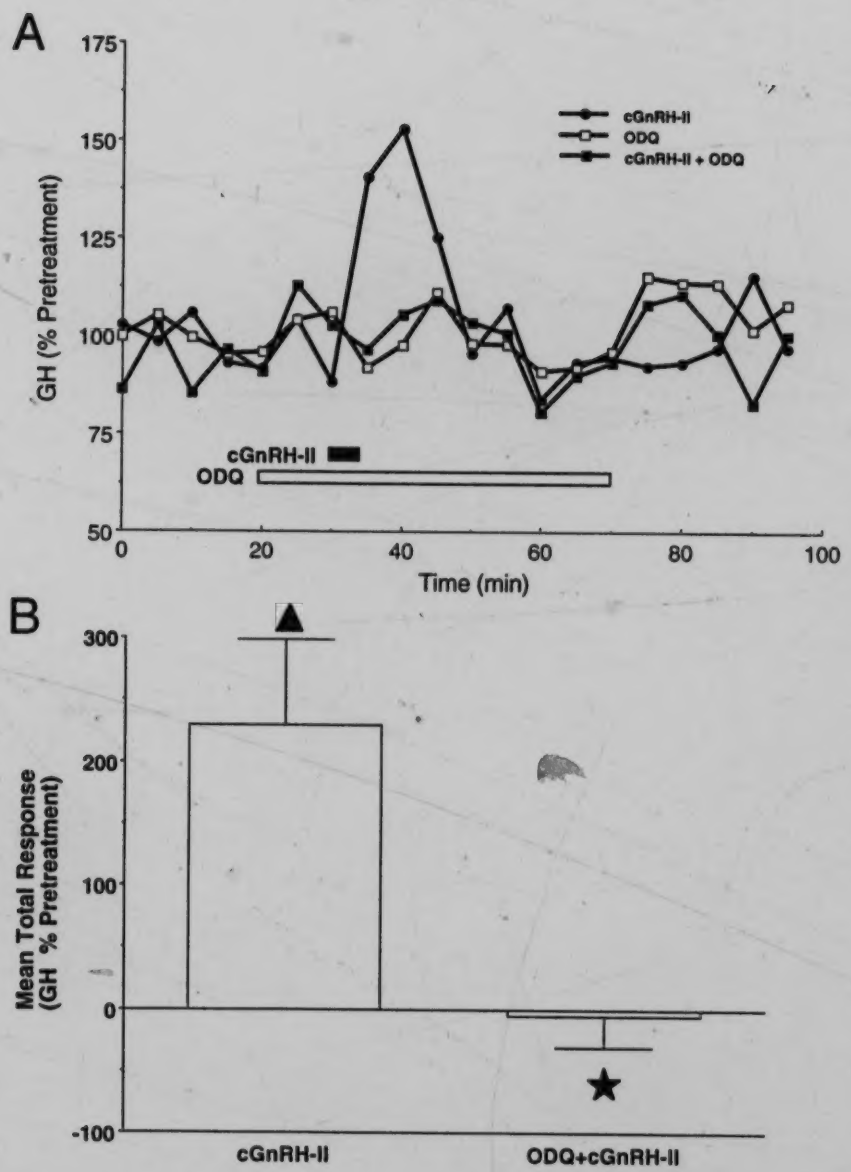
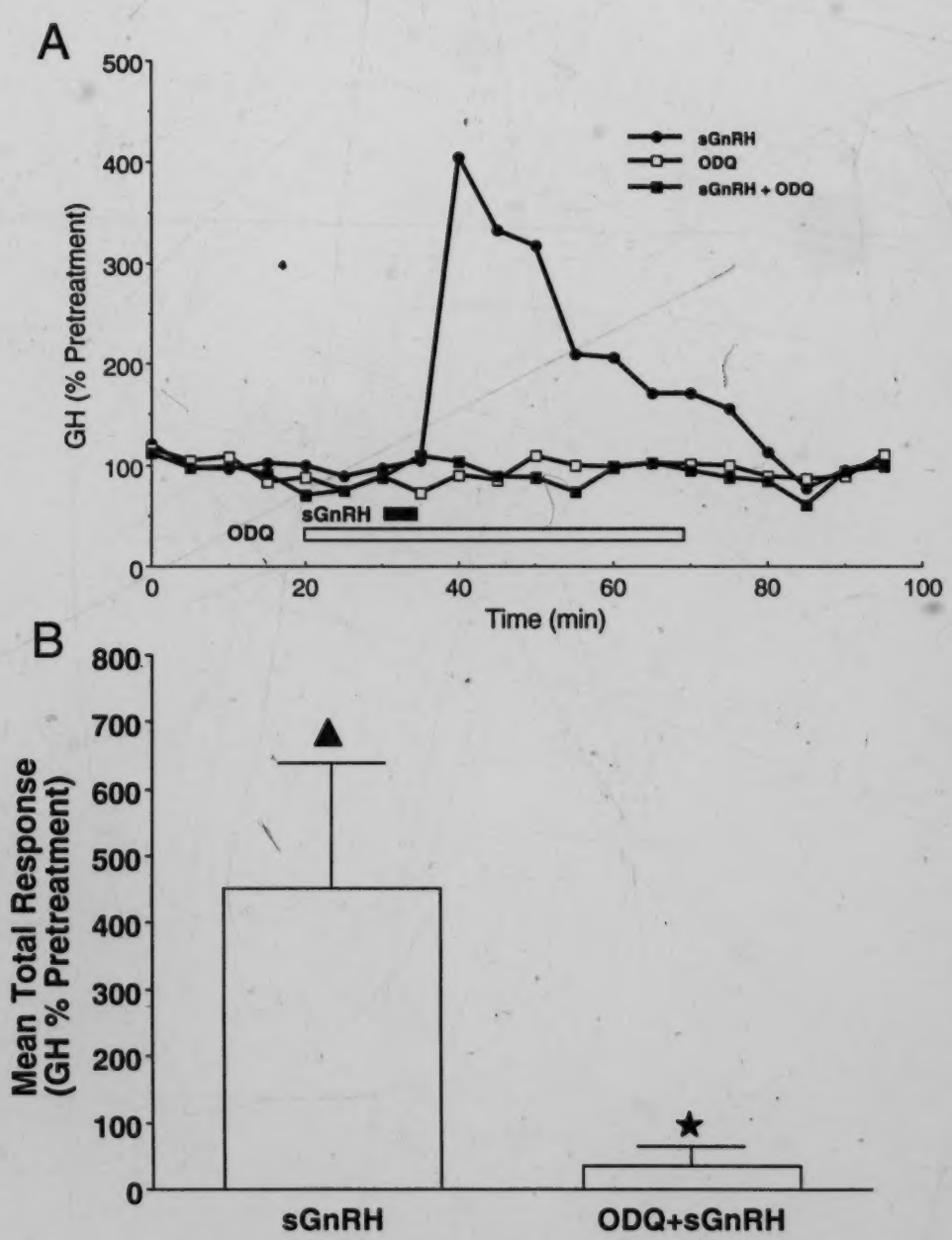

A
175
150
125
100
75
50
GH (% Pretreatment)
cGnRH-II
ODQ
cGnRH-II + ODQ
cGnRH-II
ODQ
0
20
40
60
80
100
Time (min)
B
300
200
100
0
-100
Mean Total Response
(GH % Pretreatment)
cGnRH-II
ODQ+cGnRH-II

Figure 19. sGC activity is required for the GH release response to sGnRH in cell column perifusion. **A.** Representative temporal profiles of the GH release response to sGnRH (0.1 μM) in the presence or absence of ODQ (0.1 μM). Duration of GnRH and inhibitor treatments are indicated by the horizontal bars. **B.** Total response to sGnRH in the presence and absence of ODQ. Results expressed as mean ± SEM (% pretreatment). Triangle indicates significant difference from "zero response".


A
500
400
300
200
100
0
GH (% Pretreatment)
sGnRH
ODQ
sGnRH + ODQ
sGnRH
ODQ
0
20
40
60
80
100
Time (min)
B
800
700
600
500
400
300
200
100
0
Mean Total Response
(GH % Pretreatment)
sGnRH
ODQ+sGnRH

3.3 NOS localization in pituitary cells

L-arginine is converted to NO by NOS in the presence of many cofactors, most importantly, CaM (for review see sections 1.1 and 1.2). Thus, for a cell to produce NO, it must contain some type of NOS. In order to substantiate previous hormone release data that points to the involvement of NO in the regulation of goldfish pituitary GH secretion, immunocytochemical studies were carried out with mixed pituitary cell populations in primary culture. This technique is useful because it establishes the presence of NOS, allows the further characterization of the isoform present through the use of isoform specific antibodies, and enables the identification of the specific cell types in which the enzyme reactive to the antibody are located. An antibody against the mammalian form of bNOS was chosen because bNOS is the most likely isoform of NOS to be found within the brain and its related tissues, like the pituitary. Morphologically identified somatotropes (Fig. 20A) reacted positively to the bNOS antibody (Fig. 20C). Some non-identified cells also reacted positively while others did not react at all. Overall, 23.5% of the total number of cells (210) from 10 separate photo frames reacted positively to the bNOS antibody. In addition to identified somatotropes, identified gonadotropes (Fig. 20B) also reacted positively to the bNOS antibody (Fig. 20D). An iNOS antibody was also used to perform similar experiments because the results from hormone release studies previously discussed, pharmacologically point to the participation of an iNOS-like enzyme in regulating GH secretion. Morphologically identified somatotropes (Fig. 21A) reacted positively to the iNOS antibody (Fig. 21C). This reactivity appeared to be much

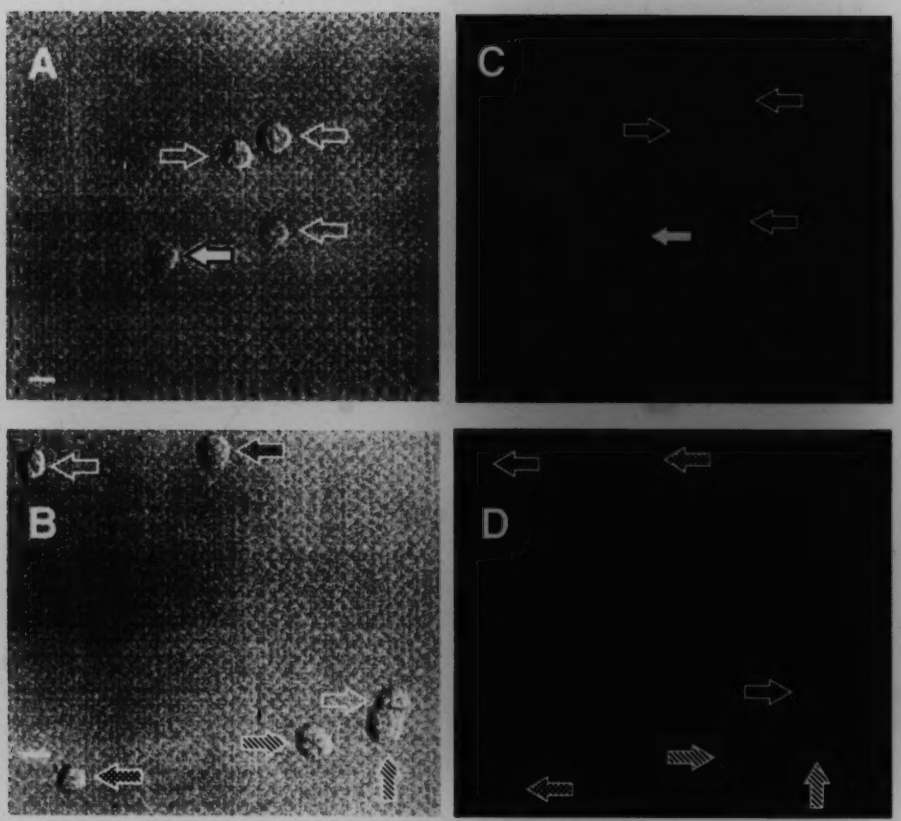


Figure 20. Presence of bNOS-like immunoreactivity in identified goldfish somatotropes and gonadotropes. **A-B.** DIC images of dispersed goldfish pituitary cells including identified somatotrope (A; filled white arrow) and gonadotropes (B; filled hatched arrows). **C-D.** bNOS immunostaining of an identified somatotrope (C) and gonadotropes (D). Clear arrows indicate unidentified cells that did not exhibit immunostaining. Filled dotted arrows indicate unidentified cells that do exhibit immunostaining. Scale bar = 10 μm.

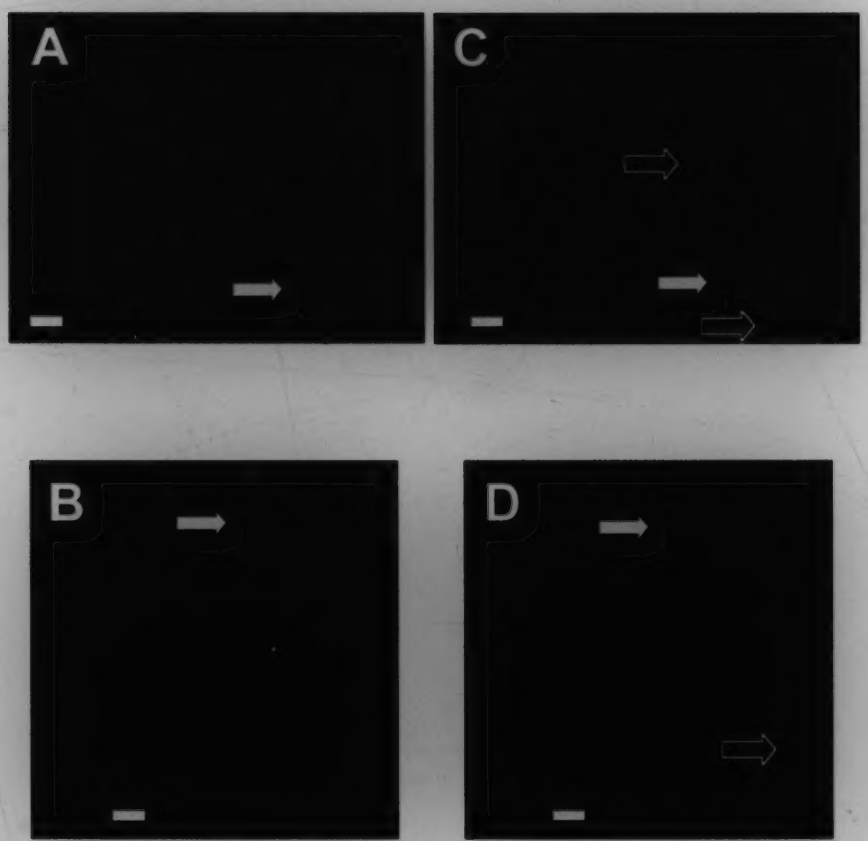


Figure 21. Presence of iNOS-like immunoreactivity in identified goldfish somatotropes and gonadotropes. **A-B.** DIC images of dispersed goldfish pituitary cells including identified somatotropes (A; clear arrows) and an identified gonadotrope (B; filled white arrow). **C-D.** iNOS immunostaining of identified somatotropes (C) and an identified gonadotrope (D). Filled white arrow in A, C = an unidentified cell. Clear arrow in B, D = an unidentified cell. Scale bar = 10 μM.

stronger than to the bNOS antibody. In addition, morphologically identified gonadotropes (Fig. 21B) also reacted positively to the iNOS antibody (Fig. 21D). Some non-identified cells reacted positively to the iNOS antibody while others did not react at all, however the ratio of non-reactive to reactive cells was much smaller with the iNOS antibody versus the bNOS antibody (percentage of cells stained positive for iNOS=80%, 8 different photo frames, 207 cells counted). No fluorescence reactivity was observed in the control group, which received no primary antibody treatment. All the above observations were consistent through ten replicate experiments. Taken together, these data strongly suggest the presence of NOS in somatotropes, gonadotropes, as well as some, but not all, other cell types in the goldfish pituitary.

3.4 NO involvement in the growth hormone release-stimulating activity of other known growth hormone secretagogues

GH release is not only stimulated by the two GnRHs in goldfish, but by many other factors as well (for review see section 1.8). Dopamine and PACAP are two other endogenous GH release-stimulating molecules that utilize a cAMP-dependent signaling system distinct from that involved in GnRH action (Wong et al., 1998; 1992). Preliminary investigations on NO involvement in mediating the actions of these two GH secretagogues were also carried out. Cells in static culture were pretreated with 7-NI (1 μM) prior to stimulation with a previously demonstrated maximal stimulatory concentration of a DA D1 agonist (SKF 38393; 1 μM; Wong et al., 1992) for 2 hours. Similar to the results using GnRHs,

7-NI had no significant effect on GH secretion stimulated by DA D1 receptor activation (Fig. 22A). In addition, 0.1-10 μM 7-NI also had no effect on the ability of a previously demonstrated, maximal stimulatory dose of PACAP-38 (0.01 μM; Wirachowsky et al., 2000) to increase GH release (Fig. 22B). To investigate the possibility that only acute, but not long-term, GH secretion may be affected by 7-NI (i.e. bNOS inhibition), cell column perifusion experiments were carried out. The GH release profile in response to 1 μM SKF 38393 was unaffected by pretreatment with 1 μM 7-NI (Fig. 23A). Furthermore, the total response to SKF 38393 was not altered by pretreatment with 7-NI (Fig. 23B). These data could support either the theory that the NOS isoform in goldfish pituitary cells is pharmacologically dissimilar to the mammalian bNOS isoform, or that NO is not involved in the DA D1- or the PACAP-stimulated GH secretion pathways.

To investigate which of these two possibilities is the more likely explanation for the results, the broader spectrum NOS inhibitor, AGH, was used. Treatment with AGH (1 mM) in static culture significantly decreased, but did not abolish the GH release response to 1 μM SKF 38393 (Fig. 24). In contrast, AGH did not reduce the acute GH response to a 5-min pulse of 1 μM SKF 38393 in perifusion experiments (Fig. 25). These data suggest that although NO is involved, it may play a limited or non-critical role in DA-D1 stimulation of GH secretion. To further examine these possibilities, effects of the iNOS specific inhibitor, 1400W (1 μM), was examined in static culture studies. The GH release response of cells in 2-hour static culture to 1 μM SKF 38393 was mildly attenuated in the presence of 1400W (Fig. 26A). Interestingly, cells stimulated

Figure 22. bNOS inhibition has no effect on DA D1- and PACAP-stimulated GH release in 2-hour static culture. **A.** Treatment with 7-NI (1 μM) did not affect basal or SKF 38393 (1 μM)-stimulated GH secretion. **B.** Treatment with 7-NI (0.1, 1, and 10 μM) did not affect basal or PACAP-38 (0.01 μM)-stimulated GH secretion. Results are presented as % basal GH (mean ± SEM). Triangle indicates significant difference from the corresponding control ($P<0.05$).

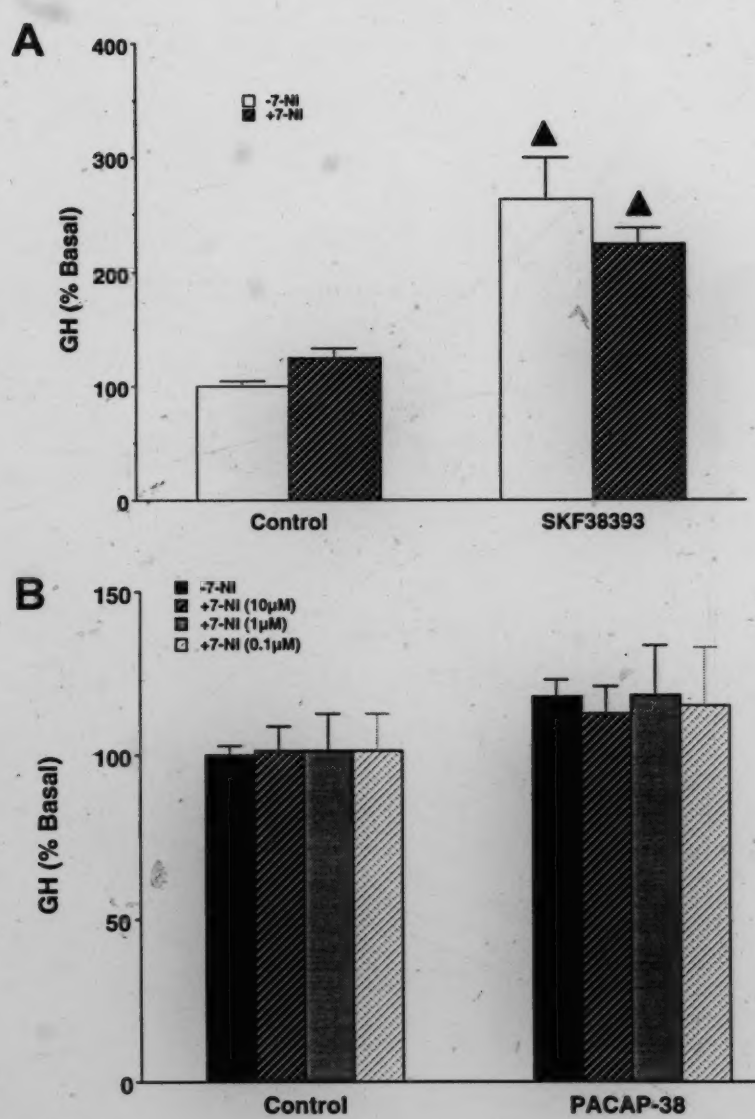
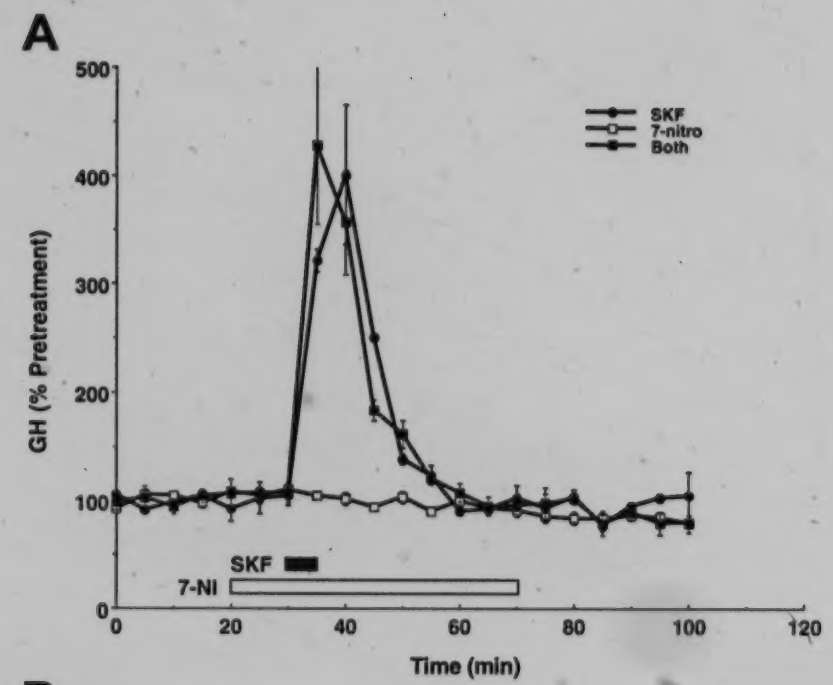
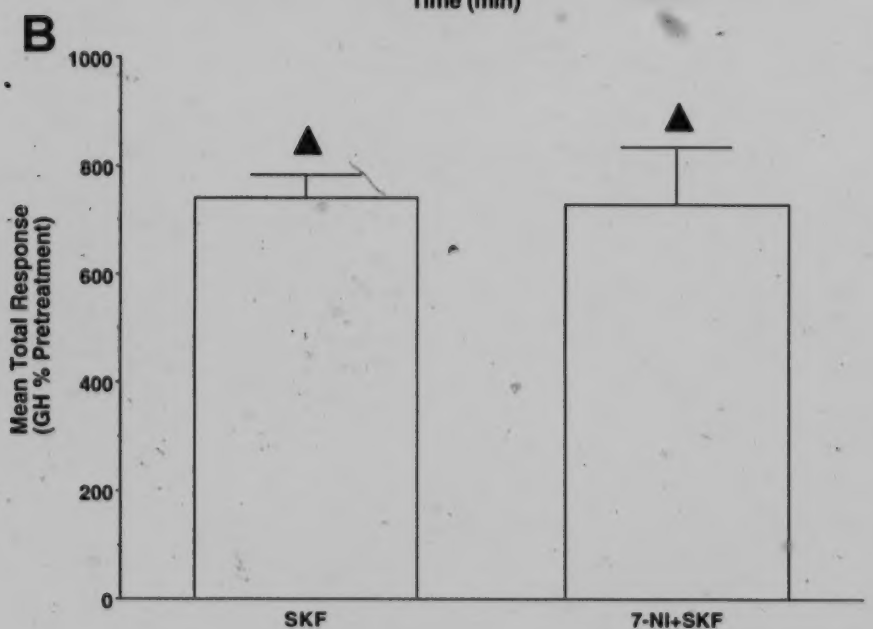

A
400
300
200
100
0
GH (% Basal)
-7-NI
+7-NI
Control
SKF38393
B
150
100
50
0
GH (% Basal)
-7-NI
+7-NI (10μM)
+7-NI (1μM)
+7-NI (0.1μM)
Control
PACAP-38

Figure 23. bNOS inhibition has no effect on DA D1-stimulated GH secretion in cell column perifusion experiments. **A.** Temporal profiles of the GH release response to SKF 38393 (1 μM; SKF) in the presence or absence of 7-NI (1 μM). Duration of SKF and 7-NI applications are indicated by the horizontal bars. **B.** Total response of cells to SKF 38393 unaffected by 7-NI. Results presented as mean ± SEM (% pretreatment). Triangle indicates significant difference from "zero response".


A
500
400
300
200
100
0
GH (% Pretreatment)
SKF
7-nitro
Both
SKF
7-NI
0
20
40
60
80
100
120
Time (min)


B
1000
800
600
400
200
0
Mean Total Response
(GH % Pretreatment)
SKF
7-NI+SKF

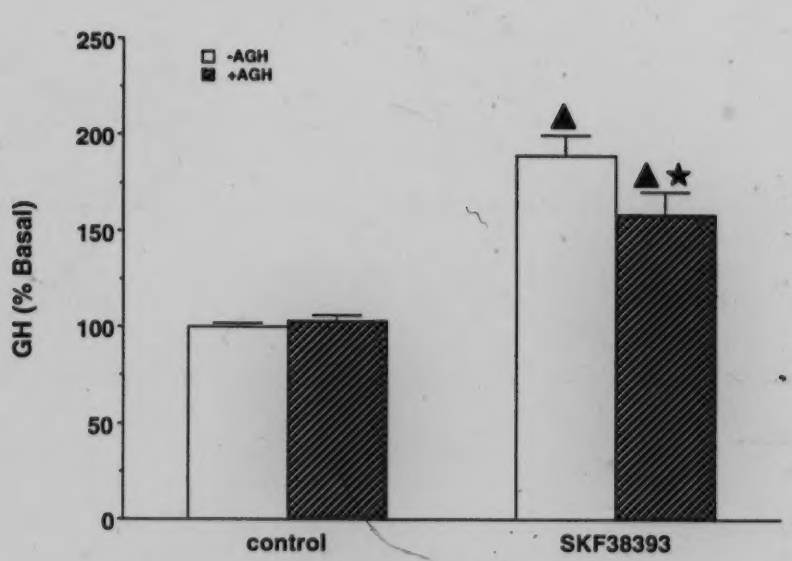
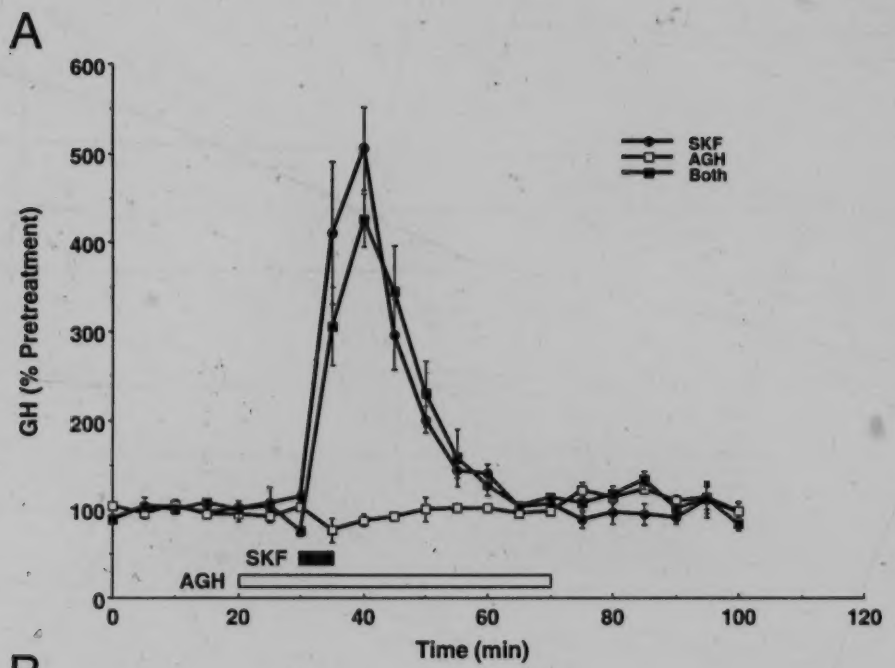


Figure 24. AGH (1 mM) significantly reduced the GH release response to SKF 38393 (1 μM) in 2-hour static culture, but has no significant effect on basal GH release. Results are presented as mean ± SEM (% basal GH). Star indicates significance from non-AGH treatment group ($P<0.05$). Triangle indicates significant difference from the corresponding control.

Figure 25. A broad spectrum NOS inhibitor has no significant effect on DA D1-stimulated GH secretion in cell column perifusion. **A.** Temporal profiles of the GH release response to SKF 38393 (1 μM; SKF) in the presence or absence of AGH (1 mM). Duration of SKF and AGH applications are indicated by the horizontal bars. **B.** Total response to SKF 38393 in the presence and absence of AGH. Results presented as mean ± SEM (% pretreatment). Triangle indicates significant difference from "zero response".


A
600
500
400
300
200
100
0
GH (% Pretreatment)
SKF
AGH
Both
SKF
AGH
0
20
40
60
80
100
120
Time (min)

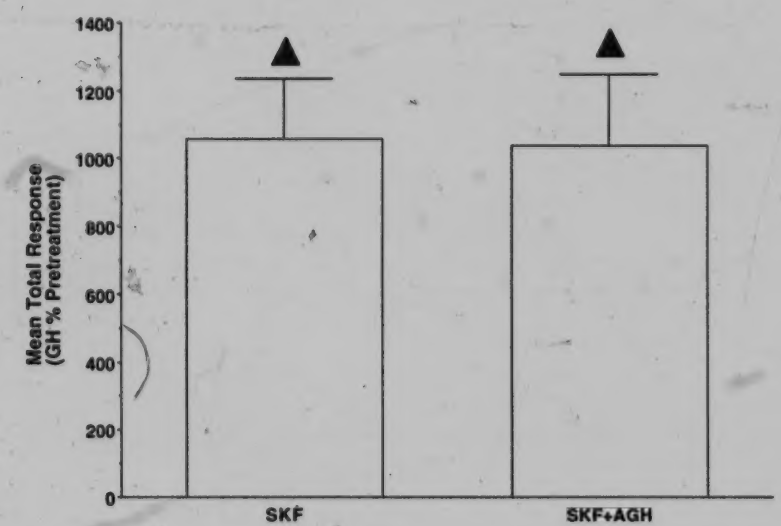
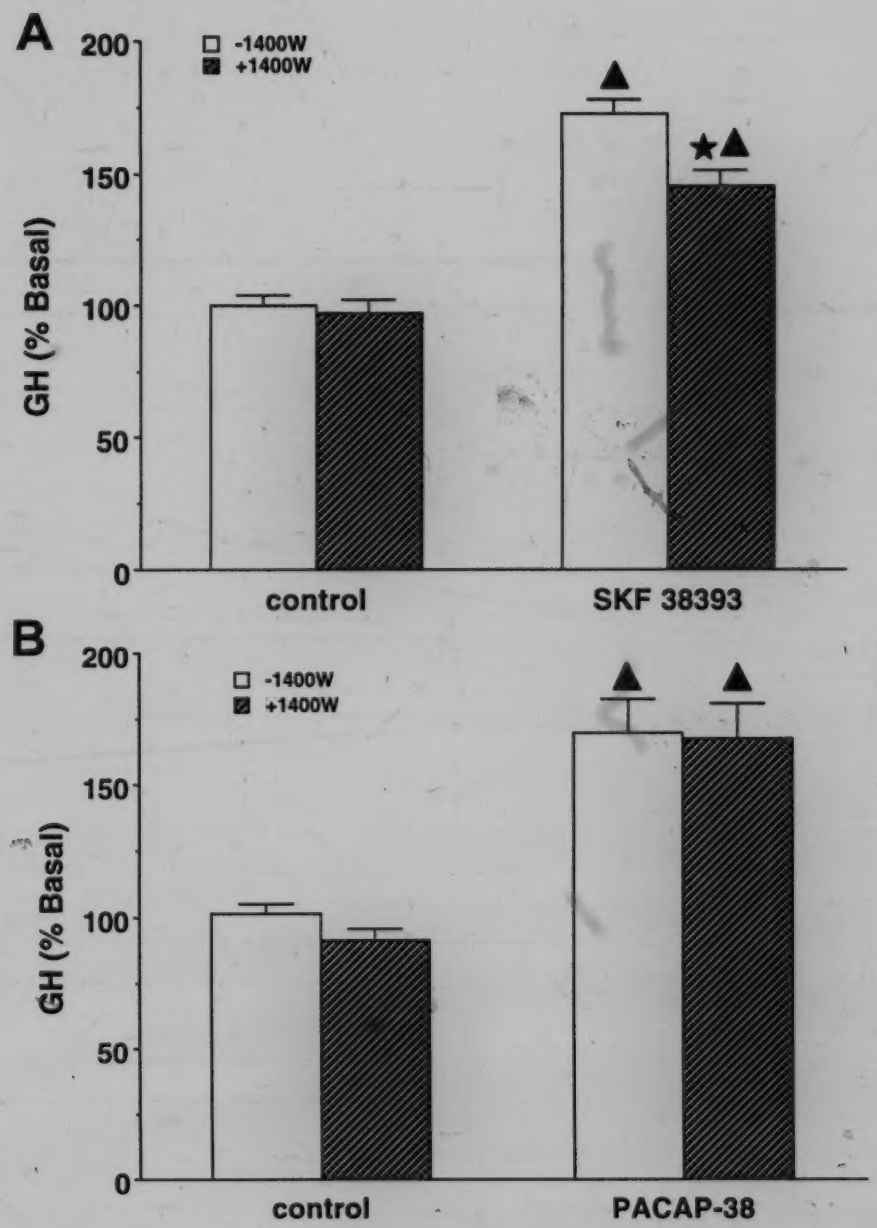

B
1400
1200
1000
800
600
400
200
0
Mean Total Response
(GH % Pretreatment)
SKF
SKF+AGH

Figure 26. iNOS inhibition reduces the GH-releasing effect of DA D1 stimulation, but does not affect PACAP-stimulated GH release in 2-hour static culture. **A.** Treatment with 1400W (1 μM) reduces the GH-release response to SKF 38393 (1 μM), but has no affect on basal GH secretion. **B.** 1400W has no affect on PACAP-38 (0.01 μM)-stimulated GH secretion. Results are presented as mean ± SEM (% basal GH). Star indicates significant difference from non-1400W-treated groups ($P<0.05$). Triangle indicates significant difference from the corresponding control.


A
-1400W
+1400W
GH (% Basal)
200
150
100
50
0
control
SKF 38393
B
-1400W
+1400W
GH (% Basal)
200
150
100
50
0
control
PACAP-38

with 0.01 μM PACAP-38 responded similarly whether or not they were exposed to 1400W (Fig. 26B). These results are consistent with a possible involvement of NO in DA-D1 stimulated GH secretion, however there results do not support a role for NO in the GH-stimulatory actions of PACAP-38.

To further investigate the signal transduction cascade that could lead to GH release following stimulation of somatotropes with DA and PACAP, and whether a possible link to NO exists, the guanylate cyclase inhibitor LY-83583 (Pandol and Schoeffield-Payne, 1990) was used. In static incubation experiments, the GH release response to both 1 μM SKF 38393 and 0.01 μM PACAP-38 were abolished in the presence of 10 μM LY-83583, while LY-83583 had no effect on basal GH release (Fig. 27). These data suggest a possible role for cGMP in generating the GH release response to both DA and PACAP. These data further indicate that the sGC/cGMP pathway may be a possible target site for the small effect that NO seems to have in the case of DA D1 stimulation. In contrast, exposure to LY 83583 did not attenuate the GH release response to SKF 38393 in perifusion experiments (Fig. 28). Taken together with the lack of inhibitory action of AGH on DA D1 agonist-induced GH release in perifusion studies (Fig. 25), these data revealed an unexpected difference in the involvement of NO and cGMP in mediating DA time-dependent action on GH release.

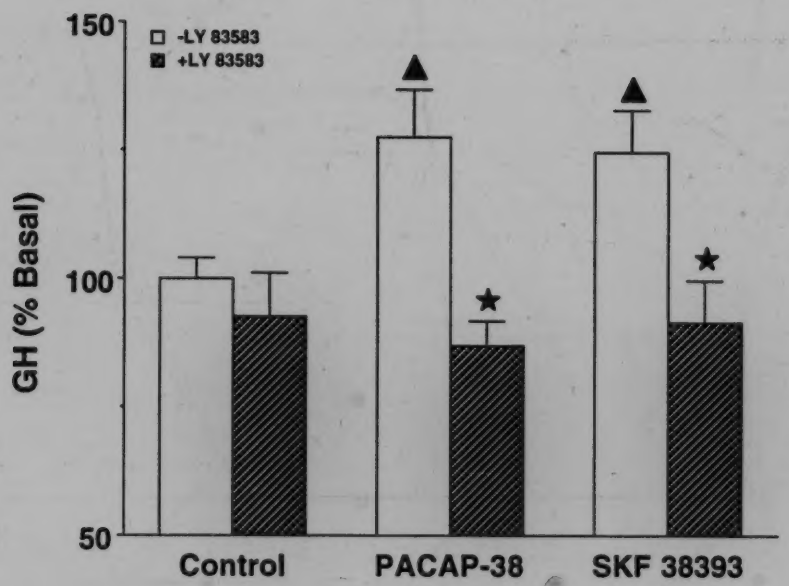
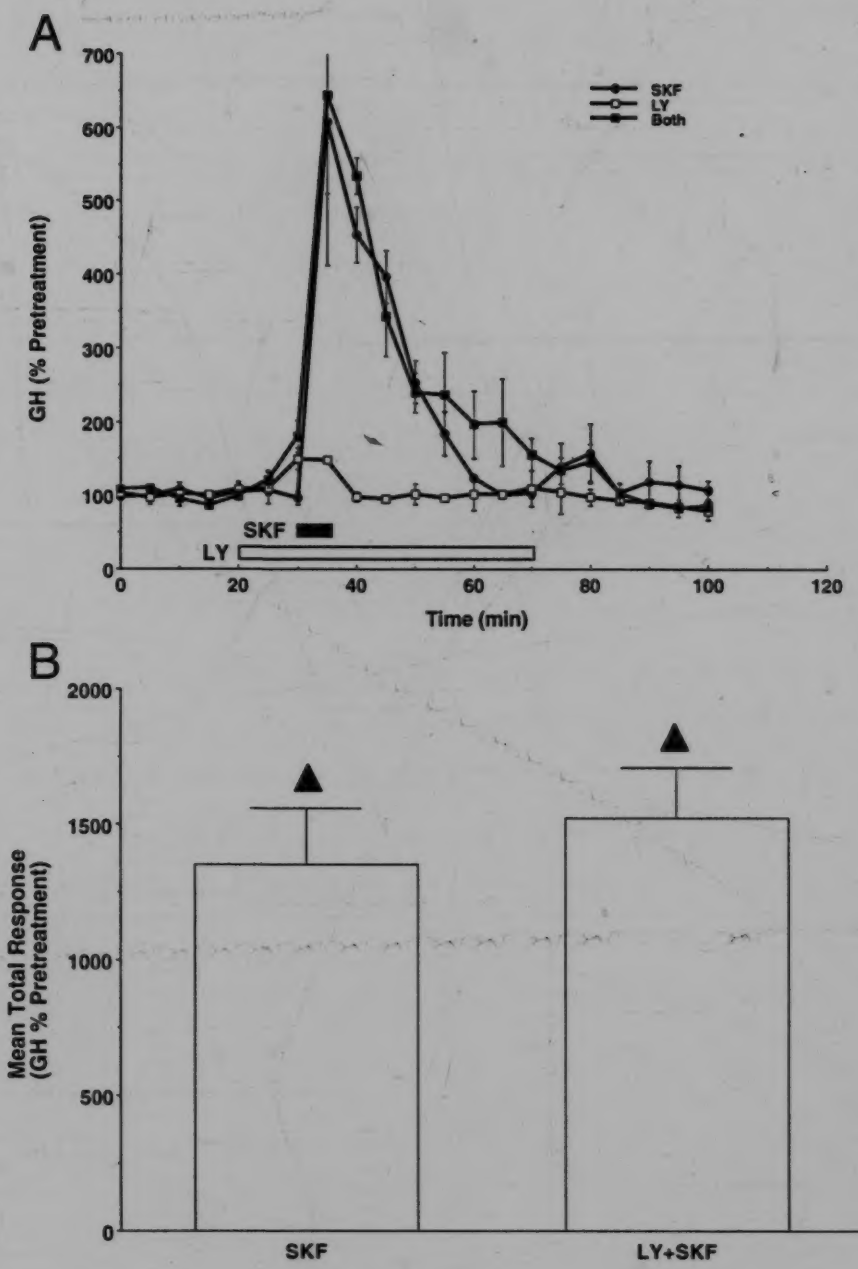


Figure 27. A guanylate cyclase inhibitor (LY 83583; 10 μM) abolishes the GH release response to both PACAP-38 (0.01 μM) and SKF 38393 (1 μM). Results are presented as mean ± SEM (% basal GH). Star indicates significant difference from non-LY 83583-treated groups ($P<0.05$). Triangle indicates significant difference from the corresponding control.

Figure 28. A guanylate cyclase inhibitor has no significant effect on DA D1-stimualted GH secretion in cell column perifusion. **A.** Temporal profiles of the GH release response to SKF 38393 (1 μM; SKF) in the presence and absence of LY 83583 (10 μM; LY). Duration of the SKF and LY treatments are indicated by the horizontal bars. **B.** Total response to SKF 38393 in the presence and absence of LY 83583. Results presented as mean ± SEM (% pretreatment). Triangle indicates significant difference from "zero response" ($P<0.05$).


A
700
600
500
400
300
200
100
0
GH (% Pretreatment)
SKF
LY
Both
SKF
LY
0
20
40
60
80
100
120
Time (min)
B
2000
1500
1000
500
0
Mean Total Response
(GH % Pretreatment)
SKF
LY+SKF

4. Discussion

Many previous studies have implicated NO as a neuroendocrine regulator that may participate at both the hypothalamic and pituitary levels to affect pituitary hormone secretion in mammals and some other vertebrate species (see sections 1.4 to 1.7). In the case of mammalian GH secretion regulation, both stimulatory and inhibitory actions of NO have been reported. In addition, the mechanisms of NO action are also contested (see section 1.6). Through a series of targeted studies, results presented in this thesis clearly demonstrate that NO is a physiological factor involved in the regulation of GH secretion at the level of the pituitary in goldfish. Studies in this thesis also addressed the mechanism of NO action, which point to the necessity of cGMP production. The revelation that NO participates in the stimulatory actions of only selected neuroendocrine factors further implies that the differential involvement of NO in cell signaling cascades may be part of the intracellular mechanisms allowing for specificity in the multifactorial control of GH secretion in the goldfish model system. Results from this thesis represent the first demonstration of NO and cGMP action in hormone secretion in teleosts.

4.1 NO stimulates growth hormone secretion

Results from hormone release studies suggest that NO can exert both long-term and short-term stimulatory influence on GH secretion in goldfish. Application of SNAP and SNP, two NO donor molecules, in 2-hour static cultures significantly stimulated GH secretion in a dose-dependent manner. Furthermore,

a 5-min pulse of a maximally stimulatory concentration of SNP (100 μM) given to cells in perifusion columns stimulated an acute and robust GH release response that lasted approximately 10-15 min. In contrast, SNAP that had been inactivated by light did not elicit a secretion response. These data agree with some results from studies on rat pituitaries suggesting a GH-stimulatory role for NO at the level of the pituitary (Pinella et al., 1999; Tena-Sempere et al., 1996; Rettori et al., 1994).

When using NO donors such as SNP, it is important to rule out the possible effects of breakdown products other than NO. This was accomplished in experiments examining the effects of an NO scavenger molecule (PTIO). PTIO is a cell permeable molecule that binds NO with high affinity (Hogg et al., 1995). With the "pre-treatment" protocol, PTIO should be able to equilibrate in and outside of cells. This protocol would allow PTIO to scavenge NO both intra- and intercellularly prior to and during SNP application. While NO is being scavenged, any other products formed by SNP while it releases NO should remain able to affect the cells. Thus, if any of the other breakdown products of SNP were causing the GH-release response seen subsequent to SNP treatment, this GH release response would be preserved in the presence of PTIO. Results obtained from this experiment clearly reveal that this is not the case and strongly implicate NO as the causative agent mediating SNP-induced GH-release (Fig. 7).

It is well known that NO is cytotoxic and can induce apoptosis in cells at high concentration (Szabolcs et al., 2001). Therefore it is also important to demonstrate that the SNP treatments used are not simply killing cells, which

would subsequently spill out any stored GH, which would in turn be measured as stimulated GH secretion. Cell viability after stimulation with SNP was evaluated, indirectly, in both cell column perifusion and static culture experiments. In perifusion, SNP treatment did not inhibit the GH release response to a DA D1 agonist (Fig. 6). In static incubation experiments, a known GH-release-inhibitory hormone (SRIH) abolished the NO donor-stimulated GH secretion (Fig. 8). These hormone release data indicate that SNP concentrations used were not cytotoxic. Furthermore, results with SRIH indicate that the NO-stimulated GH secretion pathway is subject to modulation by known neuroendocrine GH regulators.

The above results clearly demonstrate that NO can stimulate GH secretion from mixed populations of goldfish pituitary cells in primary culture. However, experiments discussed so far clearly do not address whether this stimulatory effect on GH-secretion is physiologically relevant. Nevertheless, the ability of NO to induce GH secretion from goldfish pituitary cells in primary culture provides the basis for subsequent investigations to determine if this is a physiologically relevant pathway.

4.2 NO is required for GnRH stimulation of growth hormone secretion

There is a substantial amount known about the GH secretion response to the two endogenous GnRHs in goldfish (see section 1.8). Both cGnRH-II and sGnRH bind to GnRH receptors on somatotropes. In addition, increases in $[Ca^{2+}]_c$, at least in part, by mobilization from intracellular stores, as well as by

increases in extracellular Ca^{2+} entry through voltage-sensitive calcium channels have been observed in conjunction with GnRH application (Chang et al., 2000; Johnson et al., 2000). Knowing that cNOS isoforms can be activated by increases in cytosolic Ca^{2+} (see section 1.2) and that GnRHs cause such increases, it can be predicted that the GnRH signaling cascade may involve the production of NO.

Results from studies using NOS inhibitors and NO-scavenging molecules provide evidence that NO stimulation of GH may be both physiologically relevant and even necessary for GnRH stimulation of GH release. Although addition of the NOS inhibitor selective for the mammalian bNOS isoform (7-NI) had no effect on GnRH-stimulated GH release, addition of both a more broad-spectrum NOS inhibitor (AGH) and a NOS inhibitor selective for the mammalian iNOS isoform (1400W) severely attenuated the GH release response to GnRH in static culture and perifusion studies. The fact that neither endogenous GnRH isoform was able to stimulate the release of GH in the presence of two NO scavengers with different pharmacological properties is also consistent with the involvement of NO in GnRH action. When viewed together, these data demonstrate that NO formation via the activity of a NOS enzyme located in some pituitary cells is crucial to transducing a GH-release stimulatory GnRH signal to or within somatotropes. Since NOS immunoreactivity can be detected in somatotropes as well as in gonadotropes (see section 4.6 below), whether the NOS activity that results from GnRH action at the level of the pituitary is in somatotropes

themselves, or in neighboring gonadotropes that are also stimulated by GnRH, is unclear.

Regardless of where and how NOS is activated following GnRH stimulation, the present results indicating the involvement of NO in GnRH-stimulated GH secretion is the first report of NO involvement in anterior pituitary cell secretion in teleosts. However, the participation of NO in GnRH neuroendocrine action is not without precedent. NO involvement in gonadotropin and subsequently, progesterone secretion, has been reported in the frog pituitary (Gobbetti and Zerani, 1998). Furthermore, studies on newts have reported that GnRH-induced gonadotropin secretion can be upregulated by NO in the pituitary gland (Gobbetti and Zerani, 1999). In mammals, NO has also been reported to participate in the action of GnRH stimulation of gonadotropin secretion (Moretto et al., 1998; Gonzalez and Aguilar, 1999), as well as in the regulation of GnRH neuronal activity (Mahachoklertwattana et al., 1994). In one study, SNP was reported to increase GnRH (luteinizing hormone-releasing hormone) secretion in a dose-dependent manner from hypothalamic neurons of rats in vitro (Moretto et al., 1997). In another study on rats, NO was reported to increase gonadotropin (luteinizing hormone) secretion in vitro from dispersed pituitary cells (Gonzalez and Aguilar, 1999). Whether the NOS/NO system similarly affects GnRH secretion and neuroendocrine regulation of gonadotropin in goldfish would be an interesting topic for future studies.

4.3 NO actions on growth hormone secretion are mediated by cGMP

The soluble form of the guanylate cyclase enzyme has been identified as one of NO's major intracellular targets due to its extremely high affinity for NO. It has also been reported to be one of the only enzymes activated by reaction with NO, as most others (for example, CaM kinase) are inactivated by NO. However, as discussed in section 1.3, other non-sGC/cGMP-dependent modes of action for NO also exist. Interestingly, although cGMP has been shown to stimulate GH secretion in rats (Hartt et al., 1995), the role of sGC/cGMP in mediating the controversial actions of NO in this system appears to be an inhibitory one (Tsumori et al., 1999; see section 1.6). Furthermore, NO stimulation of melanotropes is reported to be independent of sGC in *Xenopus laevis* (Allaerts et al., 2000). Thus, although it is often tempting to link NO's downstream action to sGC activation, this is not necessarily always the case.

Results from this thesis strongly implicate activation of the sGC/cGMP system as part of the mechanism by which NO stimulates GH secretion in goldfish. As in a previous study in rats (Hartt et al., 1995), goldfish pituitary cells in static culture responded to the addition of a cGMP analogue by secreting GH in quantities similar to the response to an endogenous GH secretagogue (cGnRH-II; Fig. 9). This observation leaves open the possibility that cGMP could be a mediator of GH release in response to NO. Further experiments demonstrate that cells in perifusion columns were unable to increase GH in response to an NO donor when pretreated with a NO-specific sGC inhibitor. In addition, a preliminary study has shown increases in both intracellular and

released cGMP levels in response to incubation with 100 μM SNP in 2-hour static culture. Taken together, these results strongly suggest that the sGC/cGMP signaling mechanism plays a major role in the mediation of the NO action in pituitary cells.

Studies utilizing the two endogenous isoforms of GnRH in the goldfish also implicate a physiological role of cGMP in mediating agonist-stimulated GH secretion. Both sGnRH and cGnRH-II were unable to stimulate GH secretion from cultured pituitary cells in perifusion studies in the presence of an inhibitor of sGC (Figs. 18, 19). When viewed together with the ability of AGH and 1400W to inhibit GnRH-induced GH release and the ability of ODQ to block NO-donor elicited GH secretion, these data specifically implicate a crucial role for NO production and the subsequent activation of the sGC/cGMP signaling system in the control of GH secretion in response to both GnRHs. Although not directly tested, the observation that ODQ essentially abolishes the hormone release response to NO donors and GnRHs implies that the role for other major NO signaling pathways, such as SNOs, probably do not play a prominent role in the regulation of GH secretion in the pituitary of goldfish.

In addition to supporting the argument that NO-stimulated GH release is mediated by NO action on cGMP production, the establishment that cGMP mediates GnRH-stimulated GH release is also, by itself, a significant finding. The role of GC/cGMP signaling in mediating anterior pituitary hormone release has not received as much attention as compared to other signaling cascades. Results in this thesis represent the first demonstration of the involvement of

GC/cGMP in the signal transduction leading to GH secretion in a non-mammalian vertebrate system. Only a handful of reports on cGMP involvement in pituitary hormone secretion exist in the current literature. For example, in both the frog and the newt, studies utilizing NO donors, cGMP analogues, and sGC inhibitors have reported that the sGC/cGMP system mediates the NO involvement in GnRH-induced gonadotropin secretion (Gobbetti and Zerani, 1998;1999). In addition, studies using mammalian models have reported that NO-induced LHRH secretion from hypothalamic neurons and subsequent gonadotropin secretion is mediated by the sGC/cGMP signaling system (Moretto et al., 1993). However, reports from direct studies on pituitary cells have yielded results implicating a second messenger system other than the sGC/cGMP system in mediating the NO-induced LH secretion in rats (Gonzalez and Aguilar, 1999). Thus, cGMP does not appear to be involved in all cases of NO action in the pituitary. Results from this thesis add to the growing number of studies reporting cGMP involvement in pituitary hormone secretion.

4.4 NO is not involved in unstimulated growth hormone secretion

Despite strong evidence that NO action on GH release is physiological and mediates GnRH-stimulated GH secretion, results indicate that NOS activation and NO production are likely not involved in unstimulated GH release from goldfish pituitary cells. No decrease in basal GH release was observed in all experiments performed with NOS inhibitors, NO scavengers, and GC inhibitors. These data indicate that intracellular signaling cascades mediating

stimulated exocytosis do not necessarily participate in the regulation of basal secretion. Such separate control of stimulated and unstimulated hormone secretion has been previously proposed in this model system (for review see Chang et al., 2000). It has been proposed that the cAMP/PKA, but not the PKC signaling system is involved in the regulation of basal GH secretion (Wong et al., 2001; Chang et al., 1991). Furthermore, gonadotropin secretion induced by GnRH is transduced by a PKC-dependent mechanism, while inhibition of this pathway has no effect on basal secretion (reviewed in Chang et al., 2000).

4.5 NO can be produced endogenously in goldfish pituitary cells

While all the above evidence points strongly to a physiological role for NO in the control of GH secretion in goldfish, these data do not provide direct evidence that NO is (or can be) produced. One of the well-established methods for determining if a certain cell-type has the ability to produce NO is immunocytochemistry using monoclonal or polyclonal antibodies raised against the three known isoforms of NOS. Using immunocytochemistry in conjunction with morphological identification of pituitary cell types using DIC optics, this thesis has demonstrated the presence of NOS immunoreactivity in goldfish pituitary cells.

The observation that both somatotropes and gonadotropes demonstrate immunoreactivity to both mammalian bNOS and iNOS antibodies (Fig. 20, 21) suggests that both these cell types have the potential to produce NO and further reinforces the physiological relevance of the NOS/NO system in the regulation of

pituitary cell function. In addition, this represents the first time that NOS has been localized to identified somatotropes in any animal model system. In rat pituitaries NOS has only been localized to gonadotropes and follico-stellate cells (Baht et al., 1996), although NOS mRNA has been amplified from cells contained in an enriched somatotrope fraction (Kostic et al., 2001). In the pituitaries of *Xenopus laevis*, NOS immunoreactivity was reported to be contained in nerve terminals in the pars intermedia, as well as in follico-stellate cells and melanotropes (Allaerts et al., 2000). A recent report also demonstrated the presence of bNOS activity in the neuro-intermediate lobe and the pars distalis of another teleosts species *Oreochromis niloticus* , although the cell type(s) which contain NOS reactivity has not been identified (Bordieri et al., 2001).

The localization of NOS immunoreactivity in both goldfish somatotropes and gonadotropes raises an interesting question regarding the possible sources of NO produced during GnRH stimulation of GH release. Studies presented in this thesis are unable to differentiate between intracrine, autocrine, or paracrine actions of NO generated in somatotropes and/or paracrine actions of NO generated in gonadotropes that may mediate the GH release response. The two NO scavengers used in these studies, rutin hydrate and PTIO, are both cell permeable and thus would bind NO at both intracellular and extracellular sites. However, given the highly reactive nature of NO, the effective distance it could travel may be very limited. In view of the presence of NOS immunoreactivity in somatotropes, local production of NO is likely of at least equal, if not greater

importance than production in gonadotropes, which necessitates a paracrine action to affect GH release.

While it may seem odd that both bNOS and iNOS immunoreactivity was demonstrated in goldfish somatotropes and gonadotropes, this is not the first case that both bNOS and iNOS immunoreactivity has been localized in pituitary cells. Both were demonstrated in *X. laevis* pituitary cells (Allaerts et al., 2000). It is interesting that the two reported cases of the presence of both bNOS and iNOS immunoreactivity in pituitary cells are from non-mammalian species. This may be due to the fact that antibodies used are raised against mammalian NOS isoforms and thus may have less selectivity between non-mammalian isoforms of NOS. As of now, only a partial sequence of goldfish NOS is available, and this is from macrophage cells (presumably iNOS). Whether this is the same NOS isoform that has been demonstrated by immunocytochemistry with goldfish pituitary cells by studies presented in this thesis, remains to be determined. However, it is interesting to note that a preliminary study shows that Western blotting with an antibody against a mammalian bNOS revealed the presence of at least two protein bands from hypothalamic extracts of the *O. niloticus* (Bordieri et al., 2001). These data suggest that the presence of multiple NOS isoforms in teleost tissues may not be unexpected. Preliminary Western blot studies were carried out using extracts of pituitary cells separated on electrophoresis gel and then blotted and probed with antibodies raised against specific mammalian NOS isoforms. Results have been disappointing as no immunoreactive protein was identified by this method (data not shown). While this may suggest that the

immunocytochemistry results may be misleading, this is not the first case to show NOS immunoreactivity in cytochemical studies, but no positive reactions in Western blot analysis. Studies using the pituitary cells of *X. laevis* demonstrated both iNOS and bNOS immunoreactivity in cytochemical studies, but were unable to demonstrate a positive reaction with cell lysates in Western blot analysis (Allaerts et al., 2000). It is likely that there was not enough antigenic protein recoved from cell lysates to react positively in Westen blot conditions.

There are other methods available to demonstrate NO production from cells. A nitrite assay and NO analyzers have also been used to establish and quantify NO production in vitro or in vivo. These methods are based on the fact that NO breaks down into nitrite and nitrate that are quite stable in cells. The major drawback to these methods is that very high quantities of NO must be produced to leave enough nitrite to be detected. Thus, cell cultures lasting 2-4 hours often do not accumulate detectable levels of nitrite. Therefore, to detect levels of nitrite in media, cells are often cultured for up to 3 days. Current attempts to establish NO production by nitrite assay over the same 2-hour time period used for hormone release studies were unsuccessful because levels of nitrite were well below detectable limits of the assay (data not shown). Even if NO is being produced in effective concentrations for sGC activation (nanomolar; Mayer and Hemmens, 1997), these levels are far below the detection limit of nitrite assays (typically in the 1-10 μM range).

A much newer technique was recently developed in an attempt to measure NO concentrations within cells in real time. The molecule DAF-2 was

developed as an intracellular dye sensitive to NO concentrations. Preliminary experiments with DAF-2 loaded, identified somatotropes yielded inconsistent responses. Although some cells appeared to respond with increases in fluorescence to both GnRHs some of the time, no response was observed even in positive controls trials with NO donor molecules at other times (data not shown). A probable reason for this has recently come to light. A thorough study on the properties of DAF-2 demonstrated that this dye was not only sensitive to the concentration of NO, but also to differing concentrations of Ca^{2+} and other cations, as well as to minor differences in the amount of light emitted by the microscope used (Broillet et al., 2001). This study essentially demonstrated that other conditions, that are nearly impossible to keep constant in a live cell environment, must be held constant to make DAF-2 data reliable. Thus, despite several attempts at quantifying NO production, direct proof as to the ability of goldfish pituitary cells to produce NO under hormone stimulation has yet to be established. Perhaps in future studies, direct production of [^{3}H]-citrulline from [^{3}H]-arginine, which is also an assay for NOS activity (Bredt and Snyder, 1990), can be used as a tool to study NO production. Nonetheless, the presence of NOS immunoreactivity provides a confirmation of the possibility of NO production in goldfish pituitary cells.

4.6 Is iNOS the enzyme present in goldfish pituitary cells?

This work represents the first evidence for the presence of NOS in goldfish pituitary cells. In addition, the present body of work demonstrates that the NOS

isoform present in these cells, and particularly the NOS isoform involved in mediating the hormone release processes, is immunologically and pharmacologically similar to mammalian iNOS. This is not to say that this enzyme is inducible *per se*, but that its structure and properties are such that it interacts with drugs and antibodies produced for use with the mammalian iNOS isoform. In actual fact, both immunocytochemical and hormone release studies suggest that the enzyme involved in the NO generation linked to GH release, is constitutively present. If the NOS present is an inducible form, then it would require transcription and translation to occur before its presence could be detected and NO could be produced. This is a process that takes time and therefore the acute GH release response to GnRH would have to be independent of NOS activity and NO formation. Since this is not the case, it can be concluded that whatever the exact structure of the NOS(s) present in goldfish pituitary cells, at least some of the NOS found in these cells is constitutively present and ready to be acutely activated. Whether this isoform is similar to mammalian cNOSs or the NOS identified in goldfish macrophages remains to be determined. It is also possible that the NOS present in goldfish pituitary cells is an isoform distinct from all others that have been characterized thus far. This isoform may be a new isoform that shows immunological and pharmacological similarities to mammalian iNOS while acting physiologically more similar to mammalian cNOSs. It also remains a distinct possibility that both iNOS and bNOS isoforms are present in the goldfish pituitary, as they are in the *Xenopus laevis* pituitary. However an argument can be made that even if there are two isoforms present,

at least one of them (the constitutively present one) is somewhat different from any yet identified, as it seems to respond only to drugs that are pharmacologically specific for mammalian iNOS.

4.7 NO is also involved in the regulation of dopamine-stimulated growth hormone secretion via a cGMP-mediated mechanism

In addition to GnRHs, DA is another endogenous stimulator of GH release in goldfish. DA acts on the D1 receptor on somatotropes, and through activation of the adenylate cyclase/cAMP system and/or arachidonic acid mobilization stimulates GH secretion (Chang et al., 1996). DA D1 action on somatotropes also increases $[Ca^{2+}]_c$ (W.K. Yunker and J.P. Chang unpublished).

Results with inhibitors of NOS and GC enzymes indicate that NOS and GC activation participate in the DA D1 stimulation of GH release in goldfish. These results represent the first time that these signaling components have been linked to DA action on GH cells in any vertebrate system. These results represent one of a few that have linked the DA mechanism of intracellular action to modulation of the NOS/NO pathway. Similar to observations in this thesis, DA-induced dilation of salivary ducts from the tick *Dermacentor variabilis* has been attributed to its ability to stimulate NO production (Lamoreaux et al., 2000). However, other studies have implicated a negative influence of DA on NO production. DA-induced immunosuppressive effects in response to viral infection in the central nervous system are related to its ability to suppress NO production in microglial cells (Chang and Liu, 2000). Likewise, DA-mediated inhibition of

prolactin secretion in mammals has been correlated with DA inhibition of NOS activity (Duvilanski et al., 1996) and furthermore, NO has been shown to prevent DA inhibition of prolactin secretion (Gonzalez and Aguilar, 1999). These opposing reports of DA effects on NO production may be the result of activation of different receptor subtypes present on different cells in different model systems and/or cell types.

In the present study, the properties of the NOS involved in DA D1 stimulation of GH suggests the involvement of a NOS isoform with iNOS-like pharmacological properties. As in the case for GnRH, both 1400W and AGH were able to reduce GH responses to a DA D1 agonist, but 7-NI was largely ineffective. However, the apparent involvement of NOS in DA action also differs from that for GnRH in several ways. In contrast to the complete abolishment of the GnRH-induced GH release, AGH and 1400W were only able to partially decrease the GH release response to D1 activation in static culture. In addition, these inhibitors were essentially ineffective against DA-induced responses when tested in acute GH release experiments, suggesting the presence of a time-dependent involvement of NOS/NO signaling in DA D1 action that was not observed in the case of GnRH. Consistent with results using NOS inhibitors, the guanylate cyclase inhibitor LY 83583 was able to inhibit the GH release response to a DA D1 agonist in static culture, but had no discernable effect on D1-activated GH release in perifusion. These results strongly suggest that NOS/NO/cGMP may only play a significant or discernable role in mediating long-term DA stimulation.

Interestingly, although AGH appeared to have modulated slightly, the rising phase of the acute GH response to DA D1 agonist application, no significant differences can be observed. Future studies should include more temporal experiments with higher time resolution (eg., 30 sec collection frequencies) to examine the role of NOS/NO in short-term DA action in greater detail. Nevertheless, when all results are viewed together, it appears that the involvement of this pathway in acute DA stimulation is very limited, if present. Time-dependent involvement of distinct intracellular signaling pathways in mediating pituitary hormone release has been reported for GnRH action on luteinizing hormone secretion in rats where metabolic processing of arachidonic acid via lipoxygenase enzyme activity has been shown to precede PKC activation (Chang et al., 1987). Similarly, CaM-kinase II has been proposed to participate in prolonged, but not acute gonadotropin response to GnRH in goldfish (reviewed in Chang and Jobin, 1994). Furthermore, NO has been demonstrated to be involved in the regulation of the acute, but not the prolonged phase of glucose-stimulated insulin secretion from rat pancreatic β-cells (Spinas et al., 1998).

Although postulation of a time-dependent involvement of NOS/NO/cGMP cascade activation is consistent with most of the observations on DA D1-stimulation of GH release contained in this thesis, this alone is not sufficient in explaining all findings. For example, how LY 83583 was able to abolish the GH response to a DA D1-agonist in static culture, while AGH and 1400W were only partially effective requires further explanation. While it is possible that the doses

of the NOS inhibitors used were insufficiently high to abolish all NOS activity, these concentrations were very effective against GnRH-induced GH release in both static incubation and perifusion studies. Perhaps, in the case of DA stimulation of GH secretion, cGMP involvement can be partially dissociated from the NO pathway, and/or linked to interactions with other known signaling systems used by DA (eg., cAMP). This and other possibilities require further investigation.

4.8 NO is not likely involved, but cGMP may be involved in PACAP stimulation of growth hormone release

PACAP is another well-documented GH secretagogue in goldfish. The PACAP signal is transduced in cells via an adenylate cyclase/cAMP pathway and PACAP is also known to increase $[Ca^{2+}]_c$ (Wirachowski et al., 2001). Reports in the literature indicate that PACAP action in smooth muscle relaxation is mediated by NO in several systems, including the duodenum (Yamamoto et al., 1999) and bronchial smooth muscle (Linden et al., 1999). In addition, the effects of PACAP on the consolidation of avoidance learning, are also mediated by NO (Telegdy and Kokavsky, 2000). These studies indicate that the involvement of NO in mediating PACAP action on GH secretion is a distinct possibility. Surprisingly, initial investigations into whether NO may be involved in PACAP-stimulated GH secretion have yielded results suggesting that this is not the case. bNOS and iNOS specific inhibitors had no effect on GH release induced by PACAP in static culture. However these results remain to be confirmed in perifusion studies. The

more puzzling result obtained is that inhibition of guanylate cyclase abolished the GH release response to PACAP. At least two non-mutually exclusive explanations exist. NO may be involved in this pathway, but was not detected and/or cGMP may be part of an additional signal transduction mechanism by which PACAP may transduce its signal within somatotropes independent of NO. Further study into the interaction of cGMP as well as the NOS/NO system in mediating the GH release response to PACAP is required before any statements as to their interaction can be made with any degree of certainty.

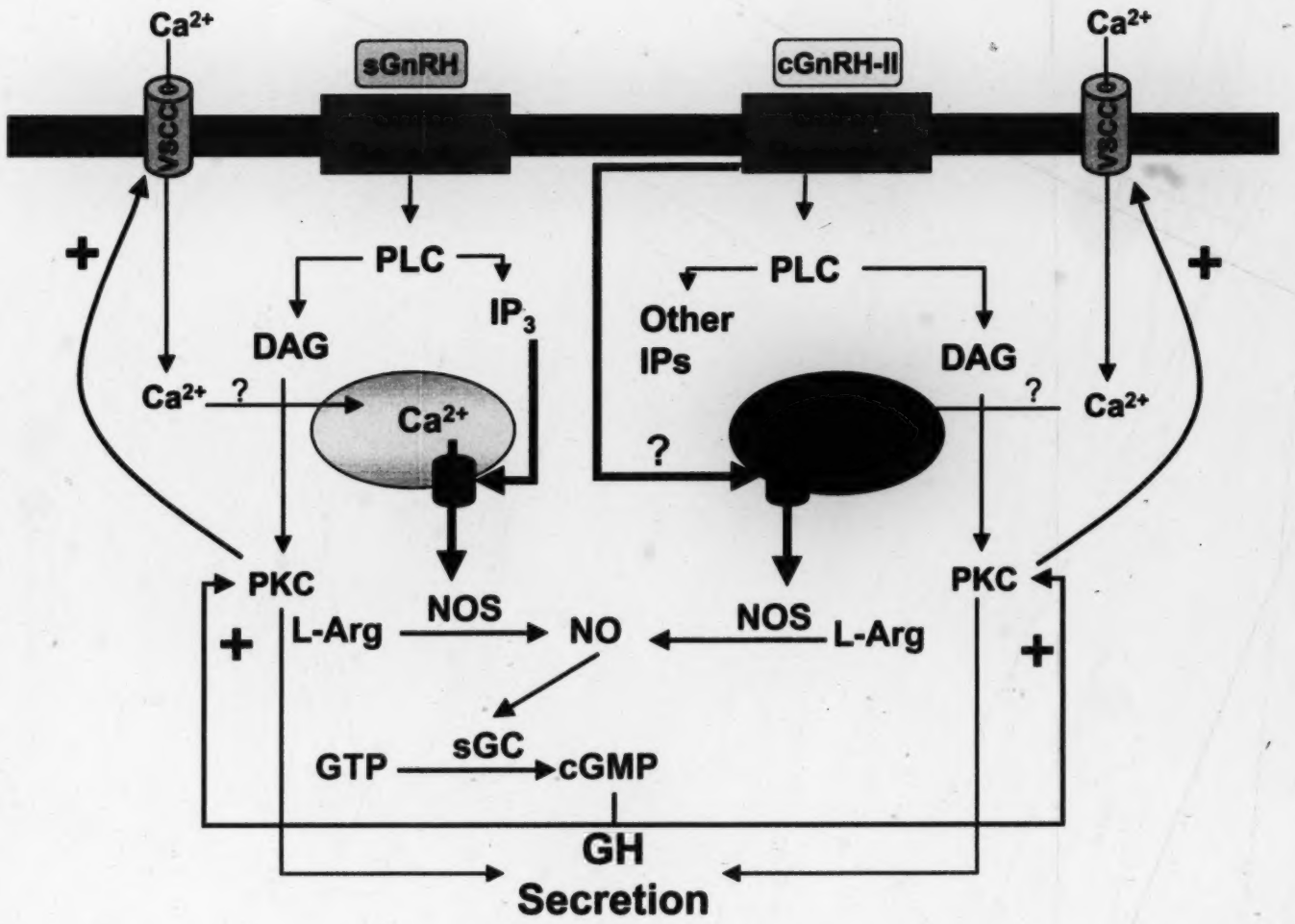
4.9 So what role does NO play in the control of growth hormone secretion?

The findings presented in this thesis have clearly shown that NO can regulate GH secretion in goldfish. In addition, these data further demonstrate the involvement of NO in agonist-stimulated GH secretion. Hypotheses as to how NO fits into the current model of GH control in goldfish can thus be generated.

4.9.1 NO in GnRH signal transduction

Evidence presented in this thesis clearly points to NO as having a crucial role in GnRH-induced GH secretion by both endogenous isoforms of GnRH. The exact order of events and interaction between GnRH receptor binding, NO formation, and the eventual secretion of GH from somatotropes is a complex one that will require further investigation. Based on what is known about GnRH signal transduction and results contained in this thesis, the following sequence of events is possible assuming that GnRH activates NOS present in somatotropes (Fig. 29).

Figure 29. Schematic representation of the possible mechanisms by which NOS activity, NO generation, and other signaling components regulate the GH secretion induced by sGnRH and cGnRH-II. Grey receptor denotes an IP_3 receptor. Blue arrows denote the IP_3-sensitive downstream mechanisms of sGnRH action. Dark purple receptor denotes a ryanodine receptor. Light purple arrows denote ryanodine-sensitive downstream mechanisms of cGnRH-II action.


Ca2+
sGnRH
cGnRH-II
Ca2+
VSCC
VSCC
+
PLC
PLC
IP3
Other
IPs
DAG
DAG
Ca2+
?
Ca2+
?
Ca2+
?
+
PKC
PKC
NOS
NOS
+
L-Arg
NO
L-Arg
+
GTP
sGC
cGMP
GH
Secretion

The Ca^{2+} sensitivity of the NOS enzyme or enzymes present in the goldfish pituitary has yet to be determined and therefore requires further study. Nevertheless, NOS enzymes that are constitutively present have been shown to be regulated by Ca^{2+} in most cases. Thus, activation of NOS may be the result of increases in cyctosolic Ca^{2+} concentration that occur subsequent to GnRH receptor binding. Previous studies in our lab have indicated that the two endogenous GnRHs can differentially regulate GH secretion through mobilization of Ca^{2+} from different intracellular stores. sGnRH, but not cGnRH-II, action utilizes an inositol trisphosphate (IP_3)-sensitive mechanism. On the other hand, only cGnRH-II-induced GH secretion is sensitive to inhibition by ryanodine (Johnson, 2000). Therefore, it is not unreasonable to assume that sGnRH and cGnRH-II activation of NOS may be mediated by mobilization of Ca^{2+} from pharmacologically distinct intracellular stores. Thus, sGnRH would activate NOS in the vicinity of IP_3-sensitive Ca^{2+}-stores, while cGnRH-II would activate NOS in the proximity of ryanodine-sensitive stores. Increased NO production then leads to activation of sGC and the formation of cGMP. The cGMP formed (and/or any downstream actions of cGMP-dependent mechanisms) could be necessary for the binding of diacylglycerol (DAG) to PKC or for the activation of PKC. It has also been determined that some PKC isoforms can be modulated by other protein kinases and cyclic nucleotides (Webb et al., 2000). PKC actions in this system have also been proposed to participate in the enhancement of Ca^{2+} entry through voltage-sensitive Ca^{2+} channels (VSCC), which plays a role in the refilling of intracellular stores and the sustaining of the GH release response

(Chang et al., 2000). Furthermore, the availability of cGMP and cGMP-dependent mechanisms may also be critical in modulating PKC influence on Ca^{2+} entry (Willmott et al., 2000). This scenario would reconcile the potential conflict created by the need to explain the previously demonstrated involvement of PKC in GnRH-stimulated GH secretion, and the crucial role of the NOS/NO/cGMP pathway revealed in this thesis.

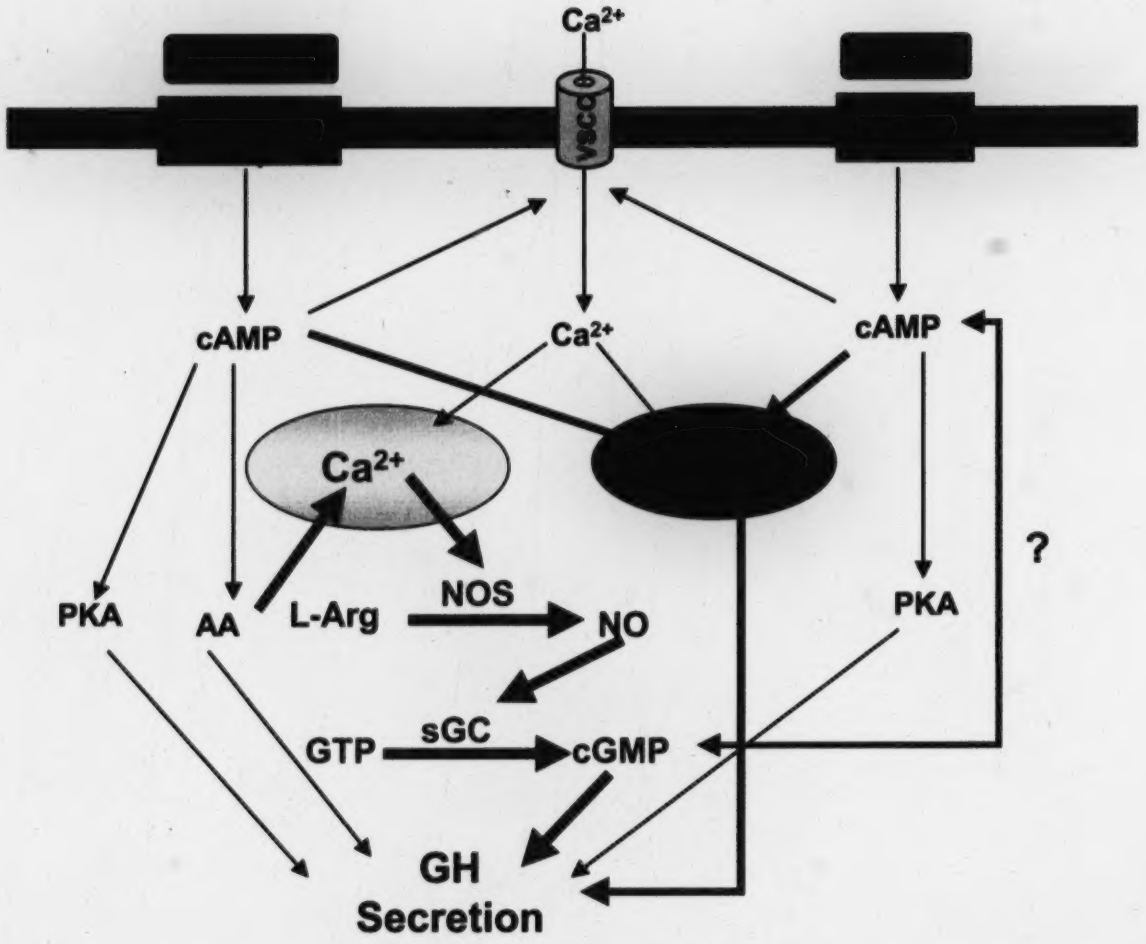
How the presence of cGnRH-II results in the differential activation of the ryanodine receptor is as of yet unknown, but the involvement of cyclic ADP-ribose is an intriguing possibility in light of its well documented links to the NOS/NO/cGMP signaling cascade (Graef et al., 1998; Willmott et al., 1996). Unfortunately, results from preliminary experiments designed to determine the involvement of cyclic ADP-ribose in GnRH-stimulated GH secretion have been difficult to interpret. Application of a dose-dependent cyclic ADP-ribose antagonist alone caused a large increase in GH release, which masked the responses to GnRH and NO donors, thus making quantification difficult.

The types and number of PKC isoforms present in goldfish somatotropes is presently unknown. However, at least ten isoforms have already been characterized in different animal species (Webb et al., 2000). Therefore, it remains a distinct possibility that multiple, differentially regulated PKC isoforms exist in goldfish somatotropes. This would provide a framework upon which differential regulation of GH cell functions by the two endogenous GnRHs and GnRH-stimulated, NO mediated, cGMP-dependent signaling mechanisms could be achieved.

4.9.2 NO in dopamine and PACAP signal transduction

The role NO plays in mediating GH secretion stimulated by other known endogenous GH secretagogues is less clear than with GnRH. Both DA D1- and PACAP-induced GH release are accompanied by increases in $[Ca^{2+}]_c$ and are dependent on TMB-8-sensitive intracellular Ca^{2+} stores (C. Fedorow, B. Soetaert, P.J. Davis, and J.P. Chang, unpublished). Thus, the potential for Ca^{2+}-dependent NOS activation by DA D1 and PACAP receptor stimulation in somatotropes exists. However, relative to the importance of NO in GnRH action, evidence suggests that NO plays a somewhat less crucial and/or more indirect role in DA D1-stimulated GH secretion, and does not participate at all in the PACAP-induced GH release response. PACAP is known to stimulate GH secretion via cAMP generation and DA is known to induce GH secretion through both cAMP and AA signal transduction pathways. It is possible that NO generation may be involved in one, but not both of these pathways. NO could be generated in response to NOS activity subsequent to Ca^{2+} mobilization from stores sensitive to AA and its metabolites formation, but not to cAMP-mediated Ca^{2+} signals. If the location of the NOS enzyme is restricted to the vicinity of Ca^{2+} stores sensitive to the DA signaling pathway, but not the PACAP signaling pathway, NO generation could occur only subsequent to DA- and not PACAP-induced Ca^{2+} signals. Since it is not yet known if AA plays a role in PACAP-induced GH secretion, this could provide a mechanism by which NO involvement in DA action occurs independently of PACAP (Fig.30). Interestingly, cGMP appears to be identified as a player in PACAP signal transduction. However, this

Figure 30. Schematic representation of possible mechanisms by which NOS activity, NO generation, and other signaling components selectively regulate the GH secretion induced by DA and PACAP. Blue arrows denote the NOS/NO/cGMP-dependent mechanism of DA D1 activation. Purple arrows denote the NOS/NO/cGMP-independent mechanisms of DA D1- and PACAP-mediated GH release response. Green arrow denotes the undetermined cAMP/cGMP-interaction leading to the cGMP dependency of PACAP-stimulated GH secretion.


Ca2+
VSCC
cAMP
Ca2+
cAMP
Ca2+
PKA
AA
L-Arg
NOS
NO
PKA
?
GTP
sGC
cGMP
GH
Secretion

may be the result of interactions between cAMP and cGMP, which are completely independent of NO. The possibility of NO and /or cGMP involvement in both DA and PACAP-induced GH secretion will provide an interesting topic for future studies.

4.10 Summary

Upon tackling this project, I set out to determine if NO could regulate GH secretion in goldfish. To do this, I have set out to achieve four main objectives. The first was to determine if any cells in the goldfish pituitary could produce NO. The second was to establish if exogenous NO could affect GH secretion. The third objective was to determine through what signal transduction mechanism NO acted. The final, and most complex objective was to determine if and how NO was involved in agonist-induced GH secretion by endogenous regulators. In this thesis I have presented evidence that multiple cell-types in the pituitary of goldfish, including somatotropes, have the ability to produce NO, contain physiological target sites for NO action, and utilize NO production to regulate the secretion of GH under stimulation by endogenous neuroendocrine secretagogues. This thesis has demonstrated that a molecule that until recently was thought of as a physiologically unimportant gas, has a crucial role in yet another physiological system and another animal. This work further supports the idea that NO is almost ubiquitous in nature and that "Overall, NO closely matches the importance of oxygen in sustaining life and physiological function by

executing a number of biological reactions made possible by the multiplicity of its reactivity within nearly all cells and tissues" (Gow and Ischiropoulos, 2001).

Endnotes

Portions of the results section have been published.

Uretsky A.D. and J.P. Chang. 2000. General and Comparative Endocrinology 118: 461-470.

Portions of the results have been published in abstract format.

Uretsky A.D. and J.P. Chang. 2000. 4th International Symposium on Fish Endocrinology.

Uretsky A.D. and J.P. Chang. 1999. Comparative Biochemistry and Physiology 124A Suppl.: S103.

Uretsky A.D., P.K. Kwong and J.P. Chang. 1999. Bulletin 30: 112.

Literature Cited

Alba-Roth J., O.A.Muller, J.Schopohl, and K.von Werder. Arginine stimulates growth hormone secretion by suppressing endogenous somatostatin secretion. *Journal of Clinical Endocrinology and Metabolism* 67: 1186-1189, 1988.

Allaerts W., W.Koopman, B.Verlaan, M.Buzzi, and P.Steerenberg. Endogenous production of nitric oxide and effects of nitric oxide and superoxide on melanotrope functioning in the pituitary pars intermedia of *Xenopus laevis*. *Nitric Oxide* 4: 15-28, 2000.

Beck K., W.Eberhardt, S.Frank, A.Huwiler, U.Mebmer, H.Muhl, and J.Pfeilschifter. Inducible NO synthase: role in cellular signalling. *The Journal of Experimental Biology* 202: 645-653, 1999.

Bhat G., V.B.Virendra, K.Aguan, and D.W.Brann. Evidence that brain nitric oxide synthase is the major nitric oxide synthase isoform in the hypothalamus of the adult female rat and that nitric oxide potentially regulates hypothalamic cGMP levels. *Neuroendocrinology* 64: 93-102, 1996.

Bonavera J., A.Sahu, P.Kalra, and S.Kalra. Evidence that nitric oxide may mediate the ovarion steroid-induced luteinizing hormone surge: involvement of excititory amino acids. *Endocrinology* 133: 2481-2487, 1993.

Bordieri L., G.Venturini, T.Persichini, and C.Cioni. Nitric oxide synthase in the hypothalamo-hypophyseal system of the teleosts *Oreochromis niloticus*. In: "*Perspectives in Comparative Endocrinology": Unity and Diversity* (Goos H.J., R.K.Rastogi, H.Vaudry, and R.Pierantoni, editors). Monduzi, Editore, Bologna, Italy: 509-516, 2001.

Bredt D.S. and S.L.Snyder. Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. *Procedings of the National Academy of Science.* 87: 682-685, 1990.

Broillet M., O.Randin, and J.Chatton. Photoactivation and calcium sensitivity of the fluorescent NO indicator 4,5-diaminofluorescein (DAF-2): implications for cellular NO imaging. *FEBS Letters* 491: 227-232, 2001.

Brunetti L. Nitric Oxide: A gas as a modulator of neuroendocrine secretions. *La Clinica Terapeutica* 144: 147-153, 1994.

Chang J.P., H.Cook, G.Freedman, A.Wiggs, G.Somoza, R.de Leeuw, and R.E.Peter. Use of pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish *Carassius auratus*: Initial morphological, static, and cell column perifusion studies. *General and Comparative Endocrinology* 77: 256-273, 1990.

Chang J.P., R.M.Jobin, and R.DeLeeuw. Possible involvement of protein kinase C in gonadotropin and growth hormone release from dispersed goldfish pituitary cells. *General and Comparative Endocrinology* 81: 447-463, 1991.

Chang J.P. and R.M.Jobin. Regulation of gonadotropin release in vertebrates: a comparison of GnRH mechanisms of action. In "*Perspectives in Comparative Endocrinology*" (Davey C.G., R.E.Peter, and S.S.Tobe, editors) National Research Council of Canada, Ottawa, Canada: 41-51, 1994.

Chang J.P., J.Graeter, and K.J.Catt. Dynamic actions of arachidonic acid and protein kinase C in pituitary stimulation by gonadotropin-releasing hormone. *Endocrinology* 120: 1837-1845, 1987.

Chang J.P., J.D.Johnson, F.Van Goor, C.J.H.Wong, W.K.Yunker, A.D.Uretsky, D.Taylor, R.M.Jobin, A.O.L.Wong, and J.I.Goldberg. Signal transduction mechanisms mediating secretion in goldfish gonadotropes and somatotropes. *Biochemistry and Cell Biology* 78: 1-15, 2000.

Chang J.P., J.T.Abele, F.Van Goor, A.O.L.Wong, and C.N.Neumann. Role of arachidonic acid and calmodulin in mediating dopamine D1- and GnRH-stimulated growth hormone release in goldfish pituitary cells. *General and Comparative Endocrinology* 102: 88-101, 1996.

Chang J.Y. and L.Z.Liu. Catecholamines inhibit microglial nitric oxide production. *Brain Research Bulletin* 52: 525-530, 2000.

Clementi E., M.Riccio, C.Sciorati, G.Nistico, and J.Meldolesi. The type 2 ryanodine receptor of neurosecretory PC12 cells is activated by cyclic ADP-ribose. *Journal of Biological Chemistry* 271: 17739-17745, 1996.

Curran R., F.Ferrari, P.Kispert, J.Stadler, D.Stuehr, R.Simmons, and T.Billiar. Nitric oxide and nitric oxide-generating compounds inhibit hepatocyte protein synthesis. *FASEB* 5: 2085-2092, 1991.

Duvilanski B., C.Zambruno, M.Lasaga, D.Pisera, and A.Seilicovich. Role of nitric oxide/cyclic GMP pathway in the inhibitory effect of GABA and dopamine on prolactin release. *Journal of Neuroendocrinology* 8: 909-913, 1996.

Fisker S., S.Nielsen, L.Ebdrup, J.N.Bech, J.S.Christiansen, E.B.Pedersen, and J.O.L.Jorgensen. The role of nitric oxide in L-arginine-stimulated growth hormone release. *Journal of Endocrinological Investigation* 22: 89-93, 1999.

Frohman L., T.Downs, and P.Chomcynski. Regulation of growth hormone secretion. *Frontiers in Neuroendocrinology* 13: 344-405, 1992.

Garvey E., J.Oplinger, E.Furfine, R.Kiff, F.Laszlo, B.Whittle, and R.Knowles. 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric oxide synthase in vitro and in vivo. *Journal of Biological Chemistry* 272: 4959-4963, 1997.

Ghigo E., J.Bellone, E.Mazza, E.Imperiale, M.Procopio, F.Valente, R.Lala, C.De Sanctis, and F.Camanni. Arginine potentiates the GHRH- but not the pyridostigmine-induced GH secretion in normal short children. Further evidence for a somatostatin suppressing effect of arginine. *Clinical Endocrinology* 32: 763-767, 1996.

Gobbetti A. and M.Zerani. In vitro nitric oxide effects on basal and gonadotropin-releasing hormone-induced gonadotropin secretion by pituitary gland of male crested newt (*Triturus carnifex*) during the annual reproductive cycle. *Biology of Reproduction* 60: 1217-1223, 1999.

Gobbetti A. and M.Zerani. Nitric oxide mediates gonadotropin-releasing hormone effects on frog pituitary. *Journal of Neuroendocrinology* 10: 407-416, 1998.

Gonzalez D. and E.Aguilar. In vitro, nitric oxide (NO) stimulates LH secretion and partially prevents the inhibitory effect of dopamine on PRL release. *Journal of Endocrinological Investigation.* 22: 772-780, 1998.

Gorbman A. Olfactory origins and evolution of the brain-pituitary endocrine system: facts and speculation. *General and Comparative Endocrinology* 97: 171-178, 1995.

Gow A. and H.Ischiropoulos. Nitric oxide chemistry and cellular signaling. *Journal of Cellular Physiology* 187: 277-282, 2001.

Graeff R.M., L.Franco, A.De Flora, and H.C.Lee. Cyclic GMP-dependent and -independent effects on the synthesis of the calcium messengers cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate. *Journal of Biological Chemistry* 273: 118-125, 1998.

Guse A.H. Cyclic ADP-ribose: a novel Ca^{2+}-mobilising second messenger. *Cell Signal* 11: 309-316, 1999.

Hartt D., T.Ogiwara, A. Ho, and C.Chik. Cyclic GMP stimulates growth hormone release in rat anterior pituitary cells. *Biochemical and Biophysical Research Communications* 214: 918-926, 1995.

Hattori R., K.Sase, H.Eizawa, K.Kosuga, T.Aoyama, R.Inoue, S.Sasayama, C.Kawai, and Y.Yui. Structure and function of nitric oxide synthases. *International Journal of Cardiology* 47: S71-S75, 1994.

Hogg N., R. Singh, J. Joseph, F. Neese, and B. Kalyanaraman. Reactions of nitric oxide with nitronyl nitroxides and oxygen: prediction of nitrite and nitrate formation by kinetic simulation. *Free Radical Research* 22: 47-56, 1995.

Holstad M., L.Jansson, and S.Sandler. Inhibition of nitric oxide formation by aminoguanidine: an attempt to prevent insulin-dependant diabetes mellitus. *General Pharmacology* 29: 697-700, 1997.

Jia L., C. Bonaventura, J. Bonaventura, J. Stanler. S-nitrosohemoglobin: a dynamic activity of blood involved in vascular control. *Nature* 380: 221-226, 1996.

Johnson, J.D. Multiple intracellular calcium stores regulate physiological functions in neuroendocrine cells. *PhD. Thesis University of Alberta*, Edmonton, Canada: 1-228, 2000.

Johnson, J.D. and J.P.Chang. Novel, thapsigargin-insensitive intracellular Ca^{2+} stores control growth hormone release from goldfish pituitary cells. *Molecular and Cellular Endocrinology* 165: 139-150, 2000.

Johnson J.D., F.Van Goor, C.J.H.Wong, J.I.Goldberg, and J.P.Chang. Two endogenous gonadotropin-releasing hormones generate dissimilar Ca^{2+} signals in identified goldfish gonadotropes. *General and Comparative Endocrinology* 116: 178-191, 1999.

Johnson J.D., F.Van Goor, R.Jobin, C.J.H.Wong, J.I.Goldberg, and J.P.Chang. Agonist-specific Ca^{2+} signaling systems, composed of multiple intracellular Ca^{2+} stores, regulate gonadotropin secretion. *Molecular and Cellular Endocrinology* 170: 15-29, 2000.

Kato M. Involvement of nitric oxide in growth hormone (GH)-releasing hormone-induced GH secretion in rat pituitary cells. *Endocrinology* 131: 2133-2138, 1992.

Klatt P., M.Schmid, E.Leopold, K.Schmidt, E.Werner, and B.Mayers. The pteridine binding site of brain nitric oxide synthase. *Journal of Biological Chemistry* 269: 13861-13866, 1994.

Knowles R.G. and S.Moncada. Nitric oxide synthases in mammals. *Biochemical Journal* 298: 249-258, 1994.

Kojima H., K.Sakurai, K.Kikuchi, S.Kawahara, Y.Kirino, H.Nagoshi, Y.Hirata, and T.Nagano. Development of a fluorescent indicator for nitric oxide based on the fluorescein chromophore. *Chemical and Pharmacological Bulletin* 46: 373-375, 1998.

Korkina K. and I. Afanas'ev. Antioxidant and chelating properties of flavonoids. *Advanced Pharmacology* 38: 151-163, 1997.

Kostic T.S., S.A.Andric, and S.S.Stojilkovic. Spontaneous and receptor-controlled soluble guanylyl cyclase activity in anterior pituitary cells. *Molecular Endocrinology* 15: 1010-1022, 2001.

Kwong P. and J.P.Chang. Somatostatin inhibition of growth hormone release in goldfish: possible targets of intracellular mechanisms of action. *General and Comparative Endocrinology* 108: 446-456, 1997.

Laing K., P.Grabowski, M.Belosevic, and C.Secombes. A partial sequence for nitric oxide synthase from a goldfish (*Carassius auratus*) macrophage cell line. *Immunology and Cell Biology* 74: 374-379, 1996.

Lamoreaux W.J., G.R.Needham, and L.B.Coons. Evidence that dilation of isolated salivary ducts from the tick *Dermacentor variabilis* (Say) is mediated by nitric oxide. *Journal of Insect Physiology* 46: 959-964, 2000.

Laszlo F., S.M.Evans, and B.J.R.Whittle. Aminoguanidine inhibits both constitutive and inducible nitric oxide synthase isoforms in rat intestinal microvasculature in vivo. *European Journal of Pharmacology* 272: 169-175, 1995.

Ledingham J. and R.Laverty. Nitric oxide synthase inhibition with N^{w}-nitro-$_{L}$-arginine methyl ester affects blood pressure and cardiovascular structure in the genetically hypertensive rat strain. *Clinical and Experimental Pharmacology and Physiology* 24: 433-435, 1997.

Linden, A., L.O.Cardell, S.Yoshihara, and J.A.Nadel. Bronchodilation by pituitary adenylate cyclase-activating peptide and related peptides. *European Respiratory Journal* 14: 443-451, 1999.

Liu Q. and S.S.Gross. Binding sites of nitric oxide synthases. *Methods in Enzymology* 268: 311-324, 1996.

Lowry M., J.I.Goldberg, and M.Belosevic. Induction of nitric oxide (NO) synthesis in murine macrophages requires potassium channel activity. *Clinical and Experimental Immunology* 111: 1-7, 1998.

Mahachoklertwattana P., S.M.Black, S.L.Kaplan, J.D.Bristow, and M.M.Grumbach. Nitric oxide synthesized by gonadotropin-releasing hormone neurons is a mediator of N-methyl-D-aspartate (NMDA)-induced GnRH secretion. *Endocrinology* 135: 1709-1712, 1994.

Marchant T., R.Fraser, P.Andrews, and R.E.Peter. The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (*Carassius auratus*) pituitary fragments in vitro. *Regulatory Peptides* 17: 41-52, 1987.

Mayer B. and B.Hemmens. Biosynthesis and action of nitric oxide in mammalian cells. *Trends in Pharmacological Sciences* 22: 477-481, 1997.

McCann S.M. The role of nitric oxide at hypothalamic and pituitary levels to alter the release of pituitary hormones: an invited commentary. *European Journal of Endocrinology* 135: 533-534, 1996.

McCann S.M., M.Kimura, A.Walczewska, S.Karanth, V.Rettori, and W.H.Yu. Hypothalamic control of gonadotropin secretion by LHRH, FSHRH, NO, cytokines, and leptin. *Domestic Animal Endocrinology* 15: 333-344, 1998.

McDonald L. and F.Murad. Nitric oxide and cyclic GMP signaling. *Society for Experimental Biology and Medicine* 1-6, 1996.

Merond E., R. Middendorff, B. Mayer, and J. Olcese. The effect of NO-donors in bovine and rat pineal cells: stimulation of cGMP and cGMP-independent inhibition of melatonin synthesis. *Journal of Neuroendocrinology* 7: 207-214, 1995.

Michel T. and O.Feron. Nitric oxide synthases: which, where, how, and why? *Journal of Clinical Investigation* 100: 2146-2152, 1997.

Moncada S., R.Palmer, and E.Higgs. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews* 43: 109-134, 1991.

Moretto M., F.Lopez, and A.Negro-Vilar. Nitric oxide regulates luteinizing hormone-releasing hormone secretion. *Endocrinology* 133: 2399-2402, 1993.

Moro M.A., R.J.Russel, S.Cellek, I.Lizasoain, Y.Su, V.M.Darley-Usmar, M.W.Radomski, and S.Moncada. cGMP mediates the vascular and platelet actions of nitric oxide: confirmation using an inhibitor of the soluble guanylyl cyclase. *Procedings of the National Acadamey of Science* 93: 1480-1485, 1996.

Müller E., V.Locatelli, and D.Cocchi. Neuroendocrine control of growth hormone secretion. *Physiological Reviews* 79: 511-604,1999.

Murad F. Cellular signaling with nitric oxide and cyclic GMP. *Brazilian Journal of Medical and Biological Research* 32: 1317-1327,1999.

Nagahama Y. Histo-physiological studies on the pituitary gland of some teleost fishes, with special reference to the classification of hormone producing cells in the adenohypophysis. *Memoirs of the Faculty of Fisheries, Hokkaido University* 21: 1-63,1973.

Oh S. and P.P.McCaslin. The iron component of sodium nitroprusside blocks NMDA-induced glutamate accumulation and intracellular Ca^{2+} elevation. *Neurochemical Research* 20: 779-84, 1995.

Pandol S.J. and M.S.Schoeffield-Payne. Cyclic GMP mediates the agonist-stimulated increase in plasma membrane calcium entry in the pancreatic acinar cell. *Journal of Biological Chemistry* 256:12846-12853.

Peng C. and R.E.Peter. Neuroendocrine regulation of growth hormone secretion and growth in fish. *Zoological Studies* 36: 78-89, 1997.

Peter R.E. and J.P.Chang. Brain regulation of growth hormone secretion and food intake in fish. In: "*Regulation of the Vertebrate Endocrine System*". (Rao P. and R.E.Peter editors). Kluwer Academic/Plenum Publishers, New York: 55-67, 1999.

Pinilla L., M.Tena-Sempere, and E.Aguilar. Nitric oxide stimulates growth hormone secretion in vitro through a calcium- and cyclic guanosine monophosphate-independent mechanism. *Hormone Research* 51: 242-247, 1999.

Rettori V., N.Belova, W.H.Yu, M.Gimeno, and S.M.McCann. Role of nitric oxide in control of growth hormone release in the rat. *Neruoimmunomodulation* 1: 195-200,1994.

Richter C., P.Ghafourifar, M.Schweizert, and R.Laffranchi. Nitric oxide and mitochondrial Ca^{2+}. *Biochemical Society Transactions* 25: 914-918,1997.

Saeij J.P.J., R.J.M.Stet, A.Groeneveld, L.B.M.Verburg-van Kemenade, W.B.van Muiswinkle, and G.F.Wiegertjes. Molecular and functional characterization of a fish inducible-type nitric oxide synthase. *Immunogenetics* 51: 339-346, 2000.

Schmidt H.H.H.W., J.S.Pollock, M.Nakane, U.Forstermann, and F.Murad. Ca^{2+}/calmodulin-regulated nitric oxide synthases. *Cell Calcium* 13: 427-434, 1992.

Sessa W.C. The nitric oxide synthase family of proteins. *Journal of Vascular Research* 31: 131-143,1994.

Sparrow J.R. Inducible nitric oxide synthase in the central nervous system. *Journal of Molecular Neuroscience* 5: 219-229,1995.

Spinas G.A., R.Laffranchi, I.Francoys, I.David, C.Richter, and M.Reinecke. The early phase of glucose-stimulated insulin secretion requires nitric oxide. *Diabetologia* 41: 292-299, 1998.

Szabolcs M., N.Ma, E.Athan, J.Zhong, M.Ming, R.Sciacca, J.Husemann, A.Albala, and P.Cannon. Acute cardiac allograft rejection in nitric oxide synthase-$2^{-/-}$ and nitric oxide synthase-$2^{+/+}$ mice: effects of cellular chimeras on myocardial inflammation and cardiomyocyte damage and apoptosis. *Circulation* 103: 2514-2520, 2001.

Telegdy G. and K.Kokavsky. The actions of pituitary adenylate cyclase activating polypeptide (PACAP) on passive avoidance learning. The role of transmitters. *Brain Research* 874: 194-199, 2000.

Tena-Sempere M., L.Pinilla, D.Gonzalez, and E.Aguilar. Involvement of endogenous nitric oxide in the control of pituitary responsiveness to different elicitors of growth hormone release in prepubertal rats. *Neuroendocrinology* 64: 146-152,1996.

Tsumori M., Y.Murakami, K.Koshimura, and Y.Kato. Endogenous nitric oxide inhibits growth hormone secretion through cyclic guanosine monophosphate-dependent mechanisms in GH_3 cells. *Endocrine Journal* 46: 779-785, 1999.

Turnbull A. and C.Rivier. Selective inhibitors of nitric oxide synthase (NOS) implicate a constitutive isoform of NOS in the regulation of interleukin-1-induced ACTH secretion in rats. *Endocrine* 5: 135-140, 1996.

Van Goor F., J.I.Goldberg, A.O.L.Wong, R.Jobin, and J.P.Chang. Morphological identification of live gonadotropin, growth-hormone, and prolactin cells in goldfish (*Carassius auratus)* pituitary-cell cultures. *Cell and Tissue Research* 276: 253-261,1994.

Vankelecom H., P.Matthys, and C.Denef. Involvement of nitric oxide in the interferon-γ-induced inhibition of growth hormone and prolactin secretion in anterior pituitary cell cultures. *Molecular and Cellular Endocrinology* 129: 157-167,1997.

Venema R.C., H.S.Sayegh, J.D.Kent, and D.G.Harrison. Identification, characterization, and comparison of the calmodulin-binding domains of the endothelial and inducible nitric oxide synthases. *Journal of Biological Chemistry* 271: 6435-6440, 1996.

Volpi R., P.Chiodera, G.Caffarri, P.P.Vescovi, L.Capretti, C.Gatti, and V.Coiro. Influence of nitric oxide on hypoglycemia- or angiotensin II-stimulated ACTH and GH secretion in normal men. *Neuropeptides* 30: 528-532, 1996.

Webb B.L., S.J.Hirst, and M.A.Giembycz. Protein kinase C isoenzymes: a review of their structure, regulation and role in regulating airways smooth muscle tone and mitogenesis. *British Journal of Pharmacology* 130: 1433-152, 2000.

Willmott N.J., K.Wong, and A.J.Strong. A fundamental role for the nitric oxide-G-kinase signaling pathway in mediatiing intercellular Ca^{2+} waves in glia. *Journal of Neuroscience* 20: 1767-1779, 2000.

Willmott N.J., J.K.Sethi, T.F.Walseth, H.C.Lee, A.M.White, and A.Galione. Nitric oxide-induced mobilization of intracellular calcium via the cyclic ADP-ribose signaling pathway. *Journal of Biological Chemistry* 271: 3699-3705, 1996.

Wirachowsky N., P.Kwong, W.K.Yunker, J.D.Johnson, and J.P.Chang. Mechanisms of action of pituitary adenylate cyclase-activating polypeptide (PACAP) on growth hormone release from dispersed goldfish pituitary cells. *Fish Physiology and Biochemistry* 23: 201-214, 2000.

Wong A.O.L., J.P.Chang, and R.E.Peter. Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the dopamine D1 receptors. *Endocrinology* 130: 1201-1210, 1992.

Wong A.O.L., M.Y.Leung, W.L.C.Shea, L.Y.Tse, J.P.Chang, and B.K.C.Chow. Hypophysiotropic action of pituitary adenylate cyclase-activating peptide (PACAP) in the goldfish: immunohistochemical demonstration of PACAP in the pituitary, PACAP stimualtion of growth hormone release from pituitary cells, and molecular cloning of pituitary type I PACAP receptor. *Endocrinology* 139: 3465-3479, 1998.

Wong C.J.H., J.D.Johnson, W.K.Yunker, and J.P.Chang. Caffeine stores and dopamine differentially require Ca^{2+} channels in goldfish somatotropes. *American Journal of Physiology* 280: R494-R503, 2001.

Xu L., J. Eu, G. Meissner, and J. Stamler. Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science* 279:234-237, 1998.

Yamada K., Z.Xu, X.Zhang, L.Gustafsson, A.Hulting, J.de Vente, H.Steinbusch, and T.Hokfelt. Nitric oxide synthase and cGMP in the anterior pituitary gland: effect of a GnRH antagonist and nitric oxide donors. *Neuroendocrinology* 65: 147-156, 1997.

Yamamoto H., A.Kuwahara, M.Fujimura, T.Maeda, M.Fujimiya. Motor activity of vascularly perfused rat duodenum. 2. Effects of VIP, PACAP27 and PACAP38. *Neurogasteroenterology and Motility* 11: 235-241, 1999.

Yunker W.K., E.K.Lee, A.O.L.Wong, and J.P.Chang. Norepinepherine regulation of growth hormone release from goldfish pituitary cells. II. Intracellular sites of action. *Journal of Neuroendocrinology* 12: 323-333, 2000.

Yunker W.K., S.Smith, C.Graves, P.J.Davis, J.E.Rivier, R.E.Peter, and J.P.Chang. Three endogenous somatostatin isoforms differentially inhibit growth hormone release in goldfish. *14th International Congress of Comparative Endocrinology* Sorrento (Napoli), Italy: 58, 2001.

Appendix A-Alternate methods

A-1 Nitrite assay

To quantify the production of NO by mixed populations of dispersed goldfish pituitary cells, an assay for the stable end product of NO production, nitrite, was performed. After a 2-hour static incubation, cells were lysed by being frozen on dry ice and were stored at -20°C until the assay was performed using the Greiss reaction. The Greiss reaction is a calorimetric assay for nitrite (Vankelecom et al., 1997). Briefly, 100 µl of samples were transferred to a 96 well microtitre plate. Equal volumes (100 µl) of 1% sulfanilamide in 2.5% phosphoric acid, and 0.1% N-naphthyl-ethylendiamine in 2.5% phosphoric acid, as well as equal volumes (25 µl) of NADH and nitrate reductase were added to each well. Contents were mixed and allowed to stand for 2 minutes, and the optical density at 540 nm of each sample was determined using an automated spectrophotometer. The approximate concentration of nitrite in the samples was determined by interpolation using a standard curve generated using known concentrations of sodium nitrite (Lowry et al., 1998).

A-2 Western blot analysis

To further confirm the presence of NOS in dispersed goldfish pituitary cells, SDS-PAGE followed by Western blot analysis were carried out. Cultured cells were lysed and and protein was collected. Briefly, cells were washed 3X with ice cold 1X D-PBS. Cells were then suspended in 500 µl of "Aub buffer" (10 mM Hepes, 5 mM EDTA, 5 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 1 mM

benzamidine) and allowed to stand on ice for 20 min. The solution was then swirled and spun at 5000 X g for 5 min. Supernatant was collected and 40 µl (8%) trichloroacetic acid was added. Precipitate were then spun at 1000 X g for 30 sec., followed by 5 washes with 1X D-PBS and resuspended in "Aub buffer" (100 µl). The protein concentration in the resultant solution was determined using a standard Bradford assay. This solution was then run on 7.5% SDS-PAGE electrophoresis gel. Protein was then transferred to nitrocellulose paper using a transfer apparatus and Western blot analysis was carried out with antibodies against both bNOS (1:1250) and iNOS (1:3000). This was followed by visualization using biotenylated goat anti-rabbit IgG treated with a streptavadin-alkaline-phosphatase conjugate followed by a color reaction with an alkaline phosphatase substrate kit (Bio-Rad).

A-3 DAF-2 imaging

Relative intracellular NO concentration was measured using the cell permeant DAF-2 DA fluorescent indicator. Cells were cultured and plated using the same methods as for immunocytochemical studies. During experiments and DAF-2 DA loading, phenyl red-free testing media was used. Cells were incubated in 10-20 µM DAF-2 DA in phenyl red-free testing media for 40-60 min at 28°C. Cells were then washed 3X with phenyl red-free testing media followed by the addition of 100 µl of phenyl red-free testing media. Cells were morphologically identified (see section 2.4) and drugs were added into the

existing media. The dye was excited at 490 nM and the emission measured at 510 nm.